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Escherichia coli O157:H7 and lactoferrin modulate immunity in cattle

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"A model is a lie that helps you see the truth."

Howard Skipper

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List of abbreviations

A/E lesions:	attaching and effacing lesions
AP-1:	Activator Protein-1
APC:	Antigen-presenting cell
bLF:	bovine Lactoferrin
Cif:	Cycle inhibiting factor
DAEC:	diffusely adherent *Escherichia coli*
EAEC:	enteroaggregative *Escherichia coli*
E. coli:	*Escherichia coli*
EDTA:	Ethylenediaminetetraacetic acid
EHEC:	enterohemorrhagic *Escherichia coli*
ELISA:	enzyme-linked immunosorbent assay
EIEC:	enteroinvasive *Escherichia coli*
EPEC:	enteropathogenic *Escherichia coli*
Esp:	*Escherichia coli* secreted protein
ETEC:	enterotoxigenic *Escherichia coli*
FDR:	False discovery rate
GADPH:	Glyceraldehyde 3-phosphate dehydrogenase
Gb3:	globotriaosylceramide
GO:	GeneOntology
HC:	hemorrhagic colitis
HCP:	hemorrhagic coli pilus
hLF:	human Lactoferrin
HUS:	hemolytic uremic syndrome
IFN-γ:	Interferon-γ
IκB:	Inhibitor of κB
IKK:	IκB kinase
IL:	Interleukin
IMS:	Immunomagnetic separation
IPA:	Ingenuity Pathway Analysis
JNK:	c-Jun N-terminal Kinase
LB:	Luria Bertani broth
LEE:	locus of enterocyte effacement
LF:	Lactoferrin
LFcinB:	Lactoferricin B
LPF:	long polar fimbriae
LPS:	lipopolysaccharide
MAPK:	Mitogen-Activated Protein Kinase
MCP-1:	Monocyte chemoattractant protein-1
MIP1-α:	Macrophage inflammatory protein 1-α
NF-κB:	Nuclear Factor-κB
NK cell:	Natural killer cell
Nle:	Non-LEE encoded effectors
PAMP:	Pathogen-associated molecular pattern

PBS:	Phosphate buffered saline
PMN:	Polymorphonuclear
PRR:	Pathogen recognition receptor
RAJ:	recto-anal junction
RNA-Seq:	RNA-Sequencing
RPS3:	ribosomal protein S3
SN:	supernatant
SRP:	Siderophore receptor and porin
STEC:	Shiga toxin-producing *Escherichia coli*
Stx:	Shiga toxins
Tir:	Translocated intimin receptor
TNF:	Tumor Necrosis Factor
TLR:	Toll Like Receptor
T3SS:	type III secretion system
TRAF2:	TNF receptor associated factor 2
UPEC:	Uropathogenic *Escherichia coli*
VTEC:	verocytotoxin-producing *Escherichia coli*

Chapter I: Literature review

1. Introduction

Human illness due to infection with enterohemorrhagic *Escherichia coli* (EHEC) is frequently occurring in developed countries (9652 cases in Europe in 2004) (Stein 2005). The clinical manifestations can range from mild symptoms such as abdominal convulsions and self-limited watery or bloody diarrhea, to severe cases such as hemorrhagic colitis (HC), acute renal failure and hemolytic uremic syndrome (HUS) (Sheng et al. 2006).

Consumption of contaminated meat and dairy products, as well as contaminated vegetables or water are a common source of infection (Torso et al. 2015; Buvens et al. 2011; Armstrong et al. 1996). Furthermore, direct contact with infected animals (Heuvelink et al. 2002) and person-to-person transmission (O'Donnell et al. 2002) has been described. Since infected cattle do not show clinical symptoms, their infection status is often unknown, augmenting the risk of bacterial transmission to humans. Outbreaks in the past have shown that not only EHEC put people in jeopardy of developing severe HC and HUS (characterized by thrombocytopenia, hemolytic anemia, and kidney failure), but also other seemingly harmless *E. coli* strains can acquire virulence factors after horizontal transmission, evolving into life-threatening pathogens. The outbreak of 2011 in Germany in which 3,816 cases were reported (including 54 deaths, and 845 cases of HUS) shows how readily enteroaggregative *E. coli* (EAEC) strains that do not produce Shiga toxins (Stx) can evolve into a serious threat for human health by the acquisition of an Stx2a-converting phage (Karch et al. 2012). This transduction changed an etiologic agent of diarrhea into one that also causes HC and HUS. The highly versatile nature of *Escherichia coli* makes them an interesting object for research.

This introductory chapter summarizes the current knowledge on the pathogenic *E. coli* with special focus on the *E. coli* O157:H7 serotype, which is often related to human disease. Insights into animal reservoirs of EHEC and their immune responses against the bacteria are discussed. Furthermore, a possible treatment strategy using a naturally occurring antibacterial agent, bovine lactoferrin (bLF), will be reviewed.

2. Shiga toxin-producing *Escherichia coli*

2.1 Nomenclature and taxonomy of Shiga toxin-producing *Escherichia coli*

E. coli strains can be subdivided in six categories depending on phenotypic traits, clinical features of caused disease, and specific virulence factors. The diarrheagenic *E. coli* can be split into enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC) – including enterohemorrhagic *E. coli* (EHEC)-, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper et al. 2004). In 1977, Konowalchuk et al. (1977) described for the first time a verocytotoxin that was found to be biologically and structurally similar to the Stx produced by *Shigella dysenteria* Type I (O'Brien et al. 1982). Later on, two forms of cytotoxins were described that could be found in different *E. coli* serotypes (Scotland et al. 1985). STEC or VTEC strains are characterized by the bacteriophage-mediated production of one or both of these cytotoxins, referred to as Stx1 or VT1 (first described as Shiga-like toxin I) and Stx2 or VT2 (first described as Shiga-like toxin II) (Scotland et al. 1983). The term EHEC was established to refer to strains such as *E. coli* O157:H7, that give rise to severe clinical signs such as HC, renal failure, and HUS. Many other STEC strains that actually belong to the classical EPEC serotypes, such as O26, O111, and O126 were more sporadically linked to human cases as they were found to produce Stx (Levine et al. 1987; Wade et al. 1979; Scotland et al. 1980). The classification of strains based upon serogroups is inconclusive as outbreaks with emerging or new hybrid strains demonstrate how versatile the strains are. The 2011 outbreak in Germany was an example of a highly virulent EAEC strain that was never before associated with outbreaks and had acquired the mechanism for toxin production.

Many studies have tried to correlate specific virulence factors with severity of disease: the combination of the locus of enterocyte effacement (LEE)-encoded *eae* gene for intimin and *stx2* is more frequently found in isolates from serotypes found in humans and is strongly associated with severe disease. The opposite is true for *stx1*, which is more frequently found in bacteria isolated from animals (Boerlin et al. 1999). Animals often carry STEC but do not show any clinical symptoms, demonstrating that the clinical course of disease is host associated.

The *E. coli* genome contains 4,200 to 5,500 genes, with less than 2,000 genes conserved among all strains of the species – the core genome. The variable genes, sometimes collocated on genomic islands, make up more than 90 % of the pan-genome (all genes in a group of organisms consisting of a common ancestor and all lineal descendents) and about 80 % of a typical genome. Continuous gene flux, due to horizontal gene transfer and deletions, occurred during *E. coli* divergence. This genetic plasticity accelerates the adaptation of *E. coli* to a wide range of environments and lifestyles, and allows multiple gene combinations that result in phenotypic diversification and the emergence of new hypervirulent strains (Lukjancenko et al. 2010).

Classification of strains based upon toxin types is carried out as Stxs can be divided into two types: Stx1 (almost identical to Stx from *Salmonella dysenteriae* Type 1) and Stx2, which can be subdivided into three (a,c,and d) and seven (a-g) subtypes, respectively. Stx types and subtypes exhibit significant differences in biological activity, receptor binding, and the capacity to be activated by elastase in intestinal mucus (Scheutz et al. 2012). The Stx subtypes are clinically relevant as they are associated with milder or more severe clinical cases. Stx2a was found to be highly associated with HUS (Friedrich et al. 2002). Furthermore, *in vitro* studies with Vero cells showed that Stx2a and Stx2d were at least 25 times more potent than Stx2b and Stx2c. In mice, the *in vivo* potency of Stx2b and Stx2c was found to be similar to that of Stx1, whereas Stx2a and Stx2d were 40 to 400 times more potent than Stx1 (Fuller et al. 2011). The capacity to produce Stx is encoded by genes on bacteriophages. Several common *stx* phage insertion sites have been reported in STEC genomes. Insertion site occupancy by *stx* phages depends on the host strain and on the availability of the preferred locus in the host strain (Ogura et al. 2009). Phages preferentially use one insertion site, but if this primary site is unavailable, a secondary site is selected (Serra-Moreno et al. 2007). In a majority of STEC strains, the expression of *stx* genes within lambdoid phages is believed to be largely under the control of the late promoter, pR' and the Q-antiterminator protein (Brüssow et al. 2004). The expression is usually repressed and production of Stx is preceded by prophage induction (Herold et al. 2004). Variations on the Q-gene have been proposed to influence the quantitative expression of Stx (Wagner et al. 2001). The Q-gene transcription is increased under inducing conditions allowing for increased transcription of the stx sites that are downstream of the Q-binding site (Brüssow et al. 2004). Inducing factors include nutritional stress, oxidative stress, UV-radiation, antibiotics, Ethylenediaminetetraacetic

acid (EDTA), hydrogen peroxide, heat shock and quorum sensing (Fortier & Sekulovic 2013), but Stx1 production can also be induced through low-iron (Wagner et al. 2002).

2.2 Pathogenesis

The pathogenesis of STEC strains is related to their ability to induce the formation of attaching and effacing (A/E) lesions and to produce one or two Stxs. The latter is linked to causing HC and HUS in humans (Karmali 2009). For colonization, the Stx are of less importance, although they can bind to enterocytes and subsequently influence the colonization receptors. In the next section, the most studied virulence factors are described including their contribution to pathogenesis.

2.3 Shiga toxin classification, structure, and role in pathogenesis

Stxs are very potent biological poisons as they can cause a wide variety of severe symptoms in different species. Furthermore, a single molecule of Stx may be sufficient to kill a cell (Tam & Lingwood 2007). Two immunologically distinct groups of Stxs have been described. In the early nineteen hundreds, Neisser and Shiga (Trofa et al. 1999) described Stx from *S. dysenteriae*. Eighty years later, this toxin – now called Stx1- was found in a group of *E. coli* isolates that were the cause of bloody diarrhea and other serious sequela like HC and HUS (Karmali et al. 1983; O'Brien et al. 1983). Stx2, which is highly related to Stx1 and has the same mode of action, is immunologically distinct from Stx1. These two types of Stxs are subdivided into toxin subtypes, as listed in Table 1.1. Only three types of Stx1 were described: Stx1a, Stx1c, and Stx1d. Stx1c and Stx1d are rarely found in human disease, and were associated with STEC isolated from patients with a mild disease course (Koch et al. 2001; Paton et al. 1995; Burk et al. 2003). For Stx2, seven subtypes have been described. Stx2c is also important for human disease but shows a reduced cytotoxicity on Vero cells compared to Stx2a (Paton et al. 1993). Stx2d was identified because incubation with elastase from intestinal mucus increases the Vero cell cytotoxicity of this toxin; this subtype is associated with cases of HUS (Persson et al. 2007). Stx2b is associated with mild disease (Piérard et al. 1998). Stx2e, Stx2f, and Stx2g are associated with disease in animals as pigs, pigeons and cattle, respectively (Weinstein et al. 1988; Schmidt et al. 2000; Leung et al. 2003). Stx1 has a 10-fold higher affinity for globotriaosylceramide (Gb3) receptor in cells than Stx2 (Head et al. 1991), but Stx2 has an approximately 400-fold lower LD_{50} in mice than Stx1 (Tesh et al. 1993).

Table 1.1: Toxin types and their clinical outcome

Toxin type	Linked with serious human disease	Reference(s)
Stx1a	Yes	(Riley et al. 1983)
Stx1c	No	(Koch et al. 2001; Paton et al. 1995)
Stx1d	No, less potent	(Burk et al. 2003)
Stx2a	Yes	(Riley et al. 1983)
Stx2b	No	(Piérard et al. 1998)
Stx2c	Yes, less toxic to Vero cells and mice	(Paton et al. 1993)
Stx2d	Yes, more toxic after incubation with elastase; less toxic to Vero cells	(Persson et al. 2007)
Stx2e	No, binds to GB4, associated with disease in pigs	(Weinstein et al. 1988)
Stx2f	No, first isolated from STEC form pigeons	(Schmidt et al. 2000)
Stx2g	No	(Leung et al. 2003)

(adapted from Melton-Celsa 2015)

Stxs belong to the bacterial AB_5-protein toxins and have a molecular weight of about 70 kDa (Fraser et al. 1994). In sensitive eukaryotic cells, the toxins can inhibit protein synthesis through the removal of an adenine residue from the 28S rRNA of the 60S ribosome (Endo et al. 1988). This enzymatic activity is present in the 32 kDa A-subunit (StxA) –composed of A1 and A2-, whereas the pentameric B-subunit (StxB) – which has 5 identical 7.7 kDa fragments- is important for binding to the cellular Gb3 receptor. StxB forms a doughnut-shaped structure with a central pore into which the carboxylterminus of StxA inserts (Fraser et al. 1994). After receptor binding, the toxin is taken up by endocytosis and undergoes retrograde sorting in early endosomes, in which retrograde tubules are formed in a clathrin-dependent manner. Stxs are transferred from the early endosome, directly to the trans-Golgi network and subsequently to the endoplasmatic reticulum. Finally, they use retro-translocation to translocate into the cell cytosol (Figure 1.1).

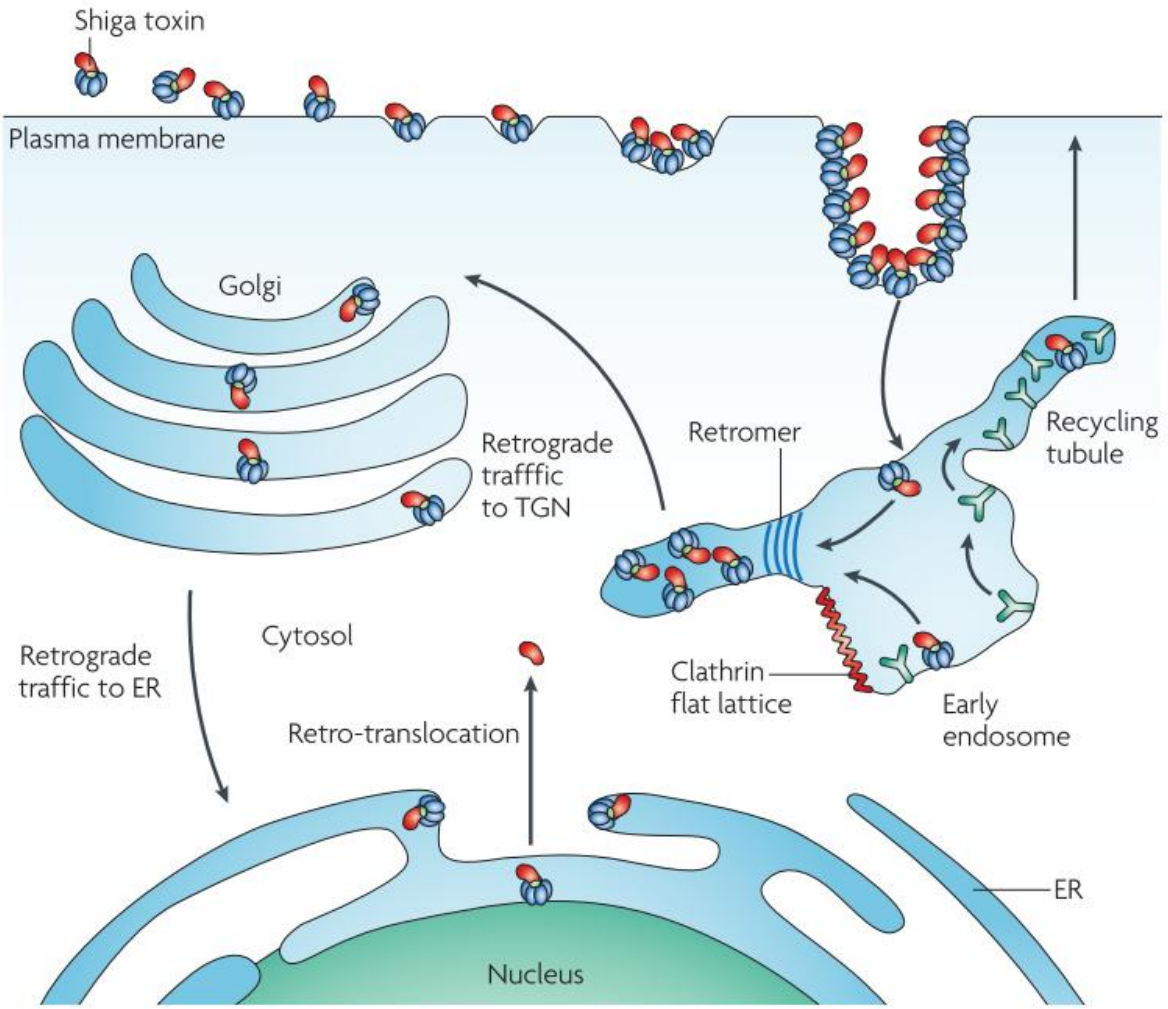

Figure 1.1: Intracellular trafficking of Stxs. Toxin binding to the plasma membrane induces endocytic invaginations, followed by retrograde sorting in early endosomes. Stxs are transported from the early endosome directly to the trans golgi network (TGN) and from there to the endplasmatic reticulum (ER) and via retro-translocation to the cytosol where the A subunit is proteolytically cleaved into an A1- and A2-fragment. (adapted from Johannes and Römer, 2010)

Once in the cytosol, the A-subunit is proteolytically cleaved into an A1- and A2-fragment. The A2-fragment remains attached to the B-subunit, whereas the A1-fragment, which is active, must avoid the proteasome until it reaches its substrate, the 60S ribosome. The protection against degradation by the proteasome is probably due to its lack of lysine residues together with its ability to refold rapidly (Johannes & Römer 2010). Stx-mediated damage to the ribosome induces a response in cells called "ribotoxic stress response", which is both proinflammatory and proapoptotic. Furthermore, an unfolded protein response as a result of the stress on the endoplasmatic reticulum, resulting in even more apoptosis has been reported (Jandhyala et al. 2012).

In order to develop HUS, translocation of the toxin into the bloodstream towards the kidney is a necessary step. In 1996, Stx translocation through the intestinal barrier was shown in intact polarized intestinal epithelial cells (Caco-2, and T84 cells) (Acheson et al. 1996). Later on, it was observed that Stx1 translocation through intestinal epithelial cells occurs via a transcellular route, whereas Stx2 uses a paracellular translocation pathway (Hurley et al. 2001; Philpott et al. 1997). In the bloodstream, Stx binds to monocytes, platelets and polymorphonuclear (PMN) leukocytes and is transferred to the kidney, inducing a prothrombotic state that contributes to the pathogenesis of HUS (Ståhl et al. 2009). Children are more sensitive to developing HUS, due to higher levels of the Gb3 receptor in the glomerular capillaries (Lingwood 1994). Furthermore, cattle lack the vascular Gb3 receptor, explaining why ruminants are symptomless carriers of *E. coli* O157:H7 (Pruimboom-Brees et al. 2000). However, they do have receptors on cortical kidney cells and crypt epithelial cells of the small and large intestine (Pruimboom-Brees et al. 2000; Hoey et al. 2002). A prolonged colonization in ruminants due to reduced epithelial shedding by the effect of Stx on crypt epithelial cells has been observed (Magnuson et al. 2000). Stxs can also bind to submucosal lymphoid cells suggesting a role for Stx in immunomodulation (Hoey et al. 2002). Furthermore, mucosal macrophages carry Stx-receptors and alter their chemokine and cytokine expression patterns in response to Stx-stimulation (Stamm et al. 2008, Menge et al. 2015).

2.4 Colonization by formation of A/E lesions

EPEC and EHEC strains must adhere to the intestinal epithelium in order to be pathogenic. This colonization requires the formation of an A/E lesion (Figure 1.2), which is characterized by destruction of nearby microvilli, intimate bacterial attachment to the apical surface and actin condensation underneath the adherent bacteria, leading to pedestal formation. The bacteria use a conserved type III secretion system (T3SS), encoded in the LEE, to form a molecular syringe (translocon) that forms a connection between the bacterium and the host cell spanning the bacterial inner and outer membrane as wells as the eukaryotic plasma membrane. This syringe translocates effector proteins as wells as gene regulators and chaperones into the target cell (Pallen et al. 2005).

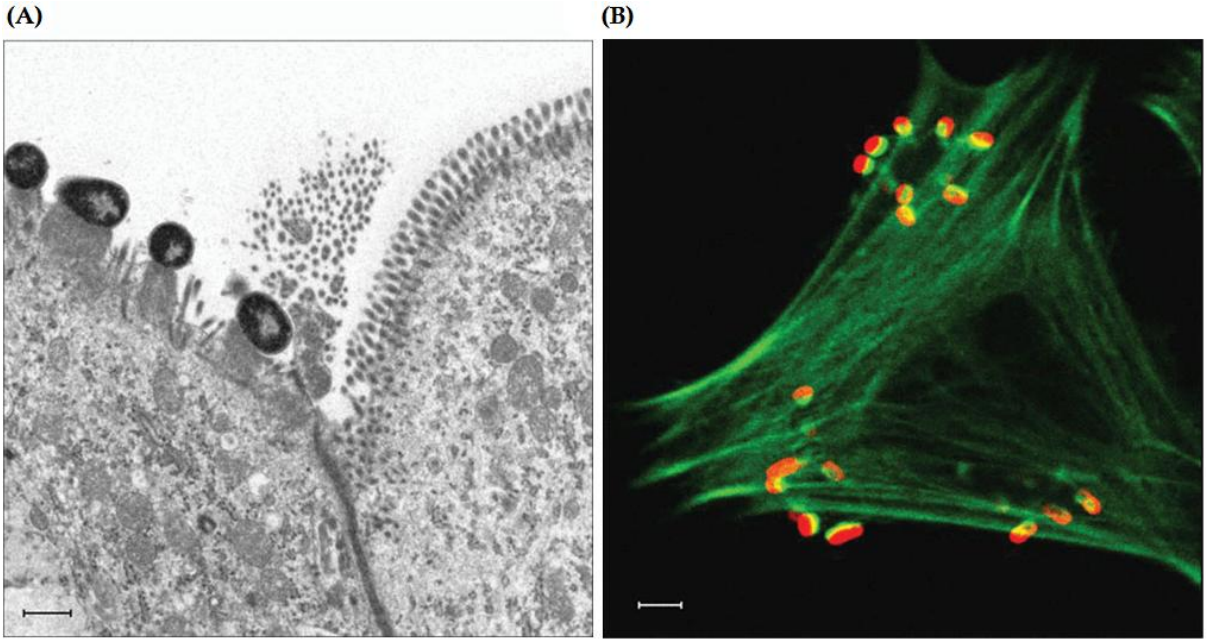

Figure 1.2: (A) Transmission electron micrograph (TEM) showing A/E lesions (pedestal formation) induced by EHEC in the colon of a neonatal calf; scale bar = 1 µm. Adapted from Stevens et al. (2002a); (B) Fluorescence micrograph showing nucleation of F-actin under EHEC strain adhering to a HeLa cell (green, F-actin detected with Oregon green514-phalloidin; red, bacteria stained with rabbit anti-O103 typing serum detected with anti-rabbit immunoglobulin-Alexa568); scale bar = 5 µm. Adapted from Stevens et al. (2002b).

In a three-step-process, EPEC and EHEC can colonize the epithelium: the first step is the initial adhesion by a not fully understood mechanism, followed by signal transduction and cytoskeletal rearrangements, for which the T3SS is required, and intimate adhesion and pedestal formation (Donnenberg et al. 1997). In the initial adhesion step, the H7 flagellin which is known to be important for motility, seems to be essential for the first adherence to the epithelium as well. Mahajan et al. (2009) have shown that the H7 flagellin which is upregulated first, functions as an adhesin.

After initial contact with the host cell, the H7 flagellin expression is downregulated while the T3SS proteins becomes upregulated. Another study suggested a role for the long polar fimbriae (LPF), as it might interact with the mucus increasing the survival of *E. coli* O157:H7 in different physiological environments (Torres et al. 2007).

The second step, mainly characterized by signal transduction and cytoskeletal reorganization of the host cell leading to the formation of an A/E lesion is instructed by the LEE. Figure 1.3 shows the T3SS apparatus encoded by LEE, resembling a molecular needle spanning the bacterial inner and outer membrane as well as the eukaryotic host membrane through which effector molecules are injected into the host cell (Pallen et al.

2005). *E. coli* secreted protein A (EspA) filaments form a transport channel between the bacteria and the host cell, transporting EspB and EspD to the host cell where they form a pore in the host cell membrane through which the bacterial effector proteins are delivered in the host cell. EspA is also important for the translocation of Tir (Translocated intimin receptor) into the host cell (Moxley 2004) which will interact with the bacterial intimin leading to an intimate adhesion of the bacterium with the host cell (DeVinney et al. 1999). Tir is injected into the host cell membrane through the needle complex and adopts a hairpin-loop structure, allowing its extracellular domain to bind to intimin (Campellone & Leong 2003). After translocation of the effector proteins, the EspA filaments are eliminated from the bacterial cell surface, which is necessary to allow intimate bacterial attachment through the intimin/Tir interaction (Frankel et al. 1998). This interaction initiates a signaling cascade, leading to polymerization of F-actin and pedestal formation which results in a strong attachment of *E. coli* to the host cell (Kaper 1998).

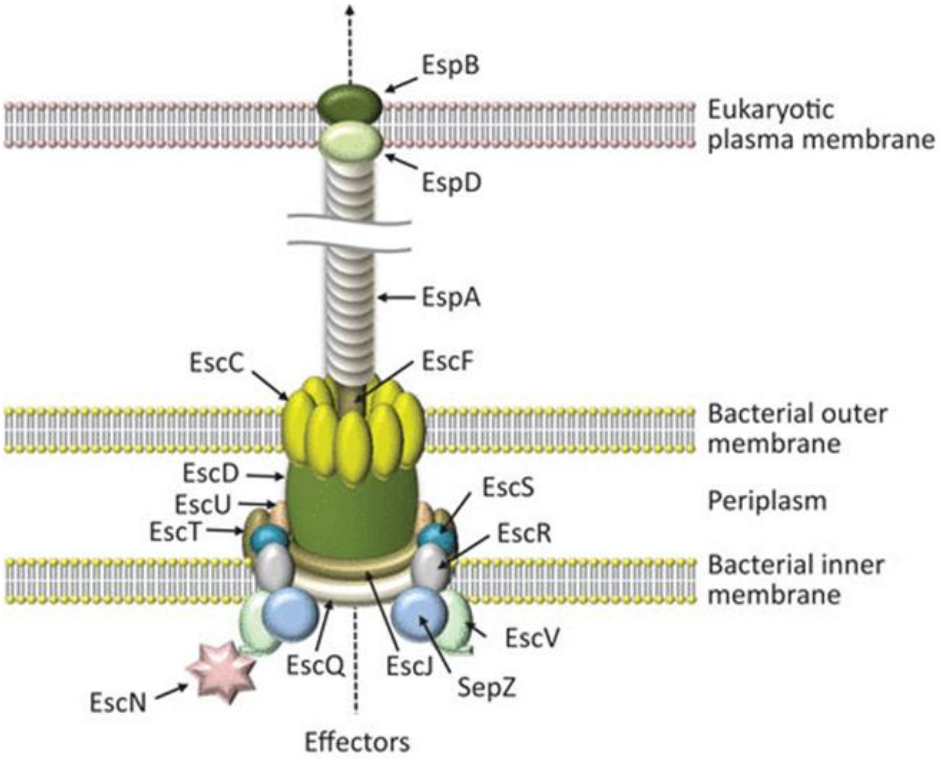

Figure 1.3: Schematic representation of the T3SS apparatus showing the organization of the LEE-encoded proteins. Adapted from Pallen et al.(2005).

This cascade of events is one of the examples which demonstrate that EHEC has evolved an ingenious strategy to attach to the host cells, inserting its own receptor and a linker molecule, but also overpowers the host cell cytoskeleton in order to create a safe

pedestal which probably impairs the removal of EHEC by immune cells. Furthermore, EHEC has developed other strategies subverting the host immune response in order to promote bacterial colonization and survival. These will be reviewed below in section 2.6.

2.5 The pO157 plasmid

All EHEC strains possess a large 90kb pO157 plasmid. These plasmids are highly conserved amongst the different *E. coli* O157:H7 strains, showing only single nucleotide polymorphisms. The complete pO157 plasmid sequence was determined by Burland et al. (1998), unraveling several possible virulence factors like enterohaemolysin, KatP, EspP, StcE and Lymphostatin (Vande Walle 2010).

2.6 Effector molecules

EHEC is an extracellular pathogen that affects intracellular host cell signaling pathways, resulting in a more efficient colonization and evasion of host immune responses. These effects are coordinated by the T3SS that injects effector molecules into the host cell. The function of these effectors ranges from inhibitors of apoptosis, influencers on inflammatory signaling pathways, to phagocytosis. The study of Wong et al. (2011) has highlighted the multifunctional nature of the effectors and their ability to participate in redundant, synergistic or antagonistic relationships, acting in a co-ordinated way on different host organelles and cellular pathways during infection. The first effectors discovered were LEE-encoded, but more recently also non-LEE encoded molecules carried on prophages or other pathogenicity islands have been described. A lineup of the best studied molecules and their effects are described below and in Figure 1.4 and Figure 1.5.

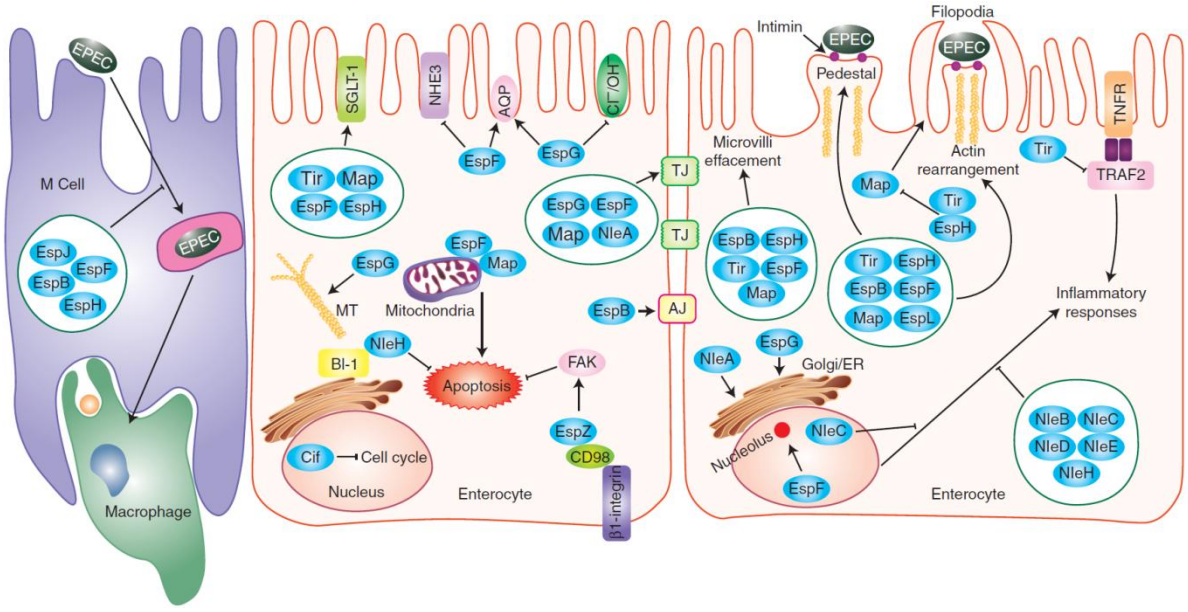

Figure 1.4: Major T3SS effectors of EPEC/EHEC and their targets within host cells to show the multifunctional nature of effectors common to EPEC and EHEC and their overlapping functions. From Law et al. (2013).

2.6.1 LEE-encoded effectors

Tir plays a role in inhibiting host innate signaling mechanisms. A study showed that transient expression of Tir from EPEC in HeLa cells inhibits Tumor Necrosis Factor (TNF)-induced Nuclear Factor-κB (NF-κB) activation by binding and degrading the cytoplasmic TRAF2 (Ruchaud-Sparagano et al. 2011). Furthermore, Tir was shown to inhibit Toll Like Receptor (TLR)-signalling and proinflammatory cytokine expression during EPEC and *Citrobacter rodentium* infection *in vitro* as well as *in vivo*, respectively. This mechanism is depending on the direct binding of Tir to SHP-1, a phosphatase that suppresses cellular immune responses by dephosphorylation of NF-κB and Mitogen-Activated Protein Kinases (MAPKs). Downregulation of SHP-1 increased the TNF and IL-6 production in EPEC-infected cells. Deletion mutants of *tir* showed enhanced NF-κB, Erk, JNK, and p38 MAPK activation when epithelial cells were infected with EPEC (Yan et al. 2012).

EspB is a natively partially folded pore-forming translocon component (Hamada et al. 2005), through which effector proteins are translocated. After pore formation, EspB is translocated into the cytosol near the site of bacterial attachment and has an effector function regulating cytoskeletal modulation (Taylor et al. 1998). EspB is known to bind host proteins α-catenin, α1-antitrypsin, and myosin that regulate the actin network leading to alterations in cell morphology (Hamada et al. 2010). The myosin-binding domain of EspB inhibits the interaction of myosins with actin, leading to microvillus

effacement (Iizumi et al. 2007). EspB is also linked to the ability of EHEC to suppress NF-κB activation and synthesis of proinflammatory cytokines. However, it remains unclear if this is due to EspB itself or to its role in the delivery of other effectors (Hauf & Chakraborty 2003).

Recent work has shown that **EspF** is one of the most multifunctional effector proteins known, comparable to a "Swiss army knife" (Holmes et al. 2010). *In vitro* studies have demonstrated that EspF from EPEC decreased the transepithelial electrical resistance, resulting in a higher permeability, as well as altered the intestinal epithelial tight junction structure (McNamara et al. 2001). Furthermore, EspF appears to be an effector of host cell death in epithelial cells by apoptosis. Its proline-rich structure suggests that it may act by binding to SH3-domains or EVH1-domains of host cell signalling proteins (Crane et al. 2001). Originally EspF was believed not to be involved in A/E lesion formation, but a more recent study has highlighted its affinity for profilin, Arp2/3, and actin leading to recruitment of junctional proteins into the pedestals, followed by the maturation of actin pedestals and the disruption of paracellular permeability (Peralta-Ramírez et al. 2008). EspF has also been linked to inhibition of apoptosis and was shown to disrupt nucleolin under regulation of mitochondrial dysfunction (Dean et al. 2010).

EspG's binding to tubulin causes localized microtubule depolymerization, resulting in actin fiber stress formation through an undefined mechanism. It is suggested that EspG contributes to the ability of A/E pathogens to establish infection through a modulation of the host cytoskeleton involving transient microtubule destruction and actin polymerization (Hardwidge et al. 2005).

EspH, first described in 2003, is translocated into the host cell using the T3SS apparatus and localizes beneath the bacterium. There, it disturbs the transient filopodium formation which is followed by a promotion of formation of actin pedestals beneath the bacterium (Tu et al. 2003).

EspZ is a control mechanism of the T3SS that prevents cytotoxicity of the host cell, when an unbalance of intracellular effector concentrations is detected. Following ectopic expression, it was found that EspZ inhibited the formation of actin pedestals as it blocked the translocation of Tir, as well as other effectors, including Map and EspF. Moreover,

during infection EspZ inhibited effector translocation following superinfection (Berger et al. 2012). EspZ contributes to epithelial cell survival by mechanisms that include the inhibition of the intrinsic apoptotic pathway (Roxas et al. 2012). This host cell cytoprotection probably enhances survival of infected enterocytes thereby promoting bacterial colonization (Wilbur et al. 2015).

Mitochondrion-associated protein (Map) features three distinct functions: (i) during initial EHEC/EPEC infection, Map is in charge of the transient formation of filopodia at the site of bacterial infection (Kenny et al. 2002); (ii) it triggers the formation of deformed mitochondria, mitochondrial swelling and damage by interfering with the host cell mechanism to maintain the mitochondrial membrane potential (Kenny & Jepson 2000); and (iii) is as essential as EspF for disrupting intestinal barrier function. While functioning independently of EspF, it alters tight junction structure and mediates these effects in the absence of mitochondrial targeting (Dean & Kenny 2004).

2.6.2 Non-LEE encoded effectors

The **Cycle inhibiting factor (Cif)**, which is encoded by a lambdoid prophage, blocks cell cycle G2/M transition and induces the formation of stress fibres through the recruitment of focal adhesions. Inhibition of the cell cycle might impair the integrity of the epithelial barrier, which eases the invasion of pathogenic bacteria into the body (Marchès et al. 2003) or prolonges their existence by blocking shedding of epithelial cells, as delayed cell death was also linked to Cif (Samba-Louaka et al. 2009).

EspJ is responsible for trans-inhibition of macrophage opsono-phagocytosis by both EPEC and EHEC, probably through targeting of a host cell molecule normally involved downstream of the phagocytic receptors FcγR and CR3, as EspJ inhibits phagocytosis mediated by these receptors (Marchès et al. 2008).

Several non-LEE encoded effectors (Nle), NleA to I, have been described. Most of their functions have been studied in EPEC strains. **NleA** (also EspI) is translocated into host cells, where it localizes to the Golgi apparatus (Gruenheid et al. 2004) and is also linked to disruption of intestinal tight junctions between epithelial cells and EPEC. This mechanism seems to be related to its inhibition of host cell protein trafficking through COPII-dependent pathways (Thanabalasuriar et al. 2011).

NleB was first identified as a secreted effector of *Citrobacter rodentium* and is conserved across all A/E pathogens (Deng et al. 2004). It was shown that NleB suppressed IκB degradation and NF-κB activation in response to TNF but not IL-1β stimulation (Newton et al. 2010). Gao et al. (2014) revealed that NleB has a conserved GlcNAc-transferase activity. They proposed the binding of NleB to host Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and subsequent O-GlcNAc modification leading to hindered TNF-receptor associated factor 2 (TRAF2)-mediated NF-κB activation. This observation is concurrent with the observations of Newton et al. (2010) that showed blocking of TNF-induced Inhibitor of κB (IκB) degradation and p65 nuclear translocation by NleB. Deletion of the *nleB* gene did not have a significant effect on the adherence of HEp-2 cells. However, in an *in vivo* model, it strongly reduced the ability of STEC O26:H11 to colonize the bovine intestinal tract (Misyurina et al. 2010).

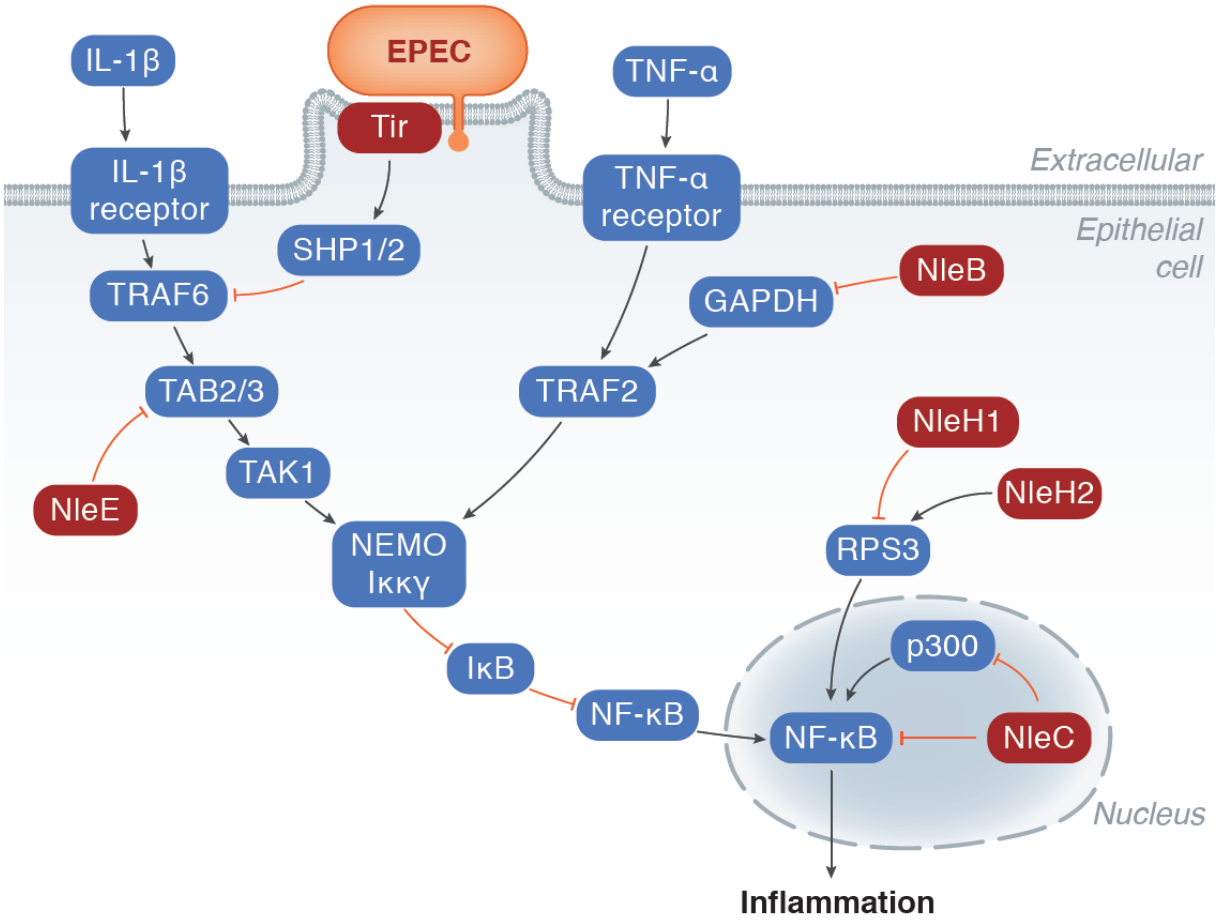

Figure 1.5: Different EPEC/EHEC effectors (shown in red) inhibit NF-κB activation by targeting components of the signaling cascade or preventing nuclear translocation of NF-κB complex components. Tir enhances the association of inhibitory SHP1 and SHP2 with TRAF6. NleE methylates TAB2/3, inhibiting TAK1 activation. NleB glycosylates GAPDH, preventing its activation of TRAF2. NleH1 inhibits the nuclear translocation of the NF-κB complex subunit, RPS3. NleH2 promotes RPS3 nuclear translocation. NleC proteolytically cleaves the p65 subunit of NF-κB. NleC also cleaves p300, an acetyltransferase that promotes p65 activity. From Santos & Finlay (2015).

NleC is a 37 kDa non-LEE encoded effector with proteolytic properties, targeting NF-κB Rel proteins for degradation, which was first identified as an effector by the LEE-encoded T3SS of *C. rodentium*. EHEC and EPEC NleC share 100% amino acid sequence similarity and are 95% similar to NleC of *C. rodentium*. NleC degrades the p65 subunit of NF-κB using zinc metalloprotease motifs. [Baruch, 2011] There is no consensus regarding the exact cleavage site of p65, as two independent research groups identified the site within the N-terminal Rel homology domain of p65, but Baruch et al. indicated the site between residue C38 and E39, whereas Yen at al. (2010) put the site between residue P10 and A11. Nevertheless, since the Rel homology domain of p65 proteins is required for binding, dimerization, and nuclear translocation, cleavage by NleC unables p65 to bind target genes and to activate transcription of inflammatory cytokines.

NleC and **NleD** are Zn^{2+}-dependent endopeptidases that specifically clip and inactivate RelA (p65) and c-Jun N-terminal Kinases (JNK), respectively, thus blocking NF-κB and Activator Protein-1 (AP-1) activation. It was shown that NleC and NleD co-operate and complement other EPEC effectors in accomplishing maximal inhibition of IL-8 secretion. This is a another example of pathogenic *E. coli* using multiple effectors to manipulate systematically the host inflammatory response signalling network (Baruch et al. 2011). A study of Yen et al. (2010) has shown that also **NleE** is necessary for inhibition of NF-κB activation as the cooperative function of NleE and NleC disrupts the NF-κB pathway and accounts for most of the immune suppression caused by EHEC/EPEC. A study in human dendritic cells and Peyer's patch dendritic cells showed that NleE can inhibit nuclear translocation of activated NF-κB and expression of proinflammatory cytokines as IL-8, IL-6 and TNF. These dendritic cells are highly present in the follicle-associated epithelium lining the gut-associated lymphoid tissue from the luminal contents of the intestinal tract. It is plausible that enteric pathogens such as EHEC, EPEC, and *Shigella sp*. have evolved to a mechanism which allows them to evade immune detection at this epithelium, promoting survival of the pathogen in the gut (Vossenkämper et al. 2010).

The highly conserved **NleF** binds to caspase-4, -8, and -9 and directly inhibits the catalytic activity of the caspases *in vitro* and in cell lysate and prevents apoptosis in HeLa and Caco-2 cells. Evasion of apoptosis helps pathogenic *E. coli* and other pathogens to take over the host cell by counteracting the cell's ability to self-destruct upon infection (Blasche et al. 2013). Furthermore, NleF induces the nuclear translocation of NF-κB p65 and the expression of IL-8 following ectopic expression and during early EPEC infection (Pallett et al. 2014).

NleH1 and **NleH2**, sharing 84% amino acid identity contribute to suppression of NF-κB activation by binding ribosomal protein S3 (RPS3), a non-Rel NF-κB subunit. The N-terminal region in NleH1 reduces the nuclear abundance of RPS3, by inhibition of IKKβ-mediated phosphorylation of RPS3 at the S209 residue, with no effect on other NF-κB signalling factors. NleH2 does not inhibit RPS3 nuclear translocation, but increases the expression of an AP-1- dependent luciferase reporter; hence it may affect a

different pathway (Gao et al. 2009). Infection studies in C57BL/6 mice showed that this effector might play a role in the persistence of the bacteria since *nleH* mutants were cleared more rapidly than the wild-type strain (Hemrajani et al. 2008). However, it was shown that neither NleH1 nor NleH2 inhibits the NF-κB activity to the same extent as NleE and NleC *in vitro* (Newton et al. 2010). Only limited data about **NleI** is available, but NleI was shown to ease the secretion of other effector molecules, like NleA, F and H (Li et al. 2006).

2.7 *E. coli* O157:H7 infection

Cattle are the main natural reservoir of EHEC, but other ruminant species such as sheep, goats, and even deer may also act as reservoirs (Ferens & Hovde 2011). Other animals like birds, swine, dogs and horses can be categorized as spillover hosts, meaning that they are susceptible to colonization and may transmit disease, but once they are no longer exposed to a source of EHEC, they do not maintain colonization. A third host category are dead-end hosts, which are incapable of transmitting EHEC naturally to other animals. They can only transmit EHEC when consumed. Examples of dead-end hosts are finfish and shellfish (Persad & LeJeune 2014). Below we focus on the main reservoir of EHEC, cattle, as well as the most important modes of transmission towards human and the effect of an infection in humans.

2.7.1 EHEC in cattle

Cattle are regarded as the main natural reservoir of STEC and EHEC, showing no clinical symptoms upon infection. Some serogroups such as *E. coli* O5, O26, and O118 are sometimes linked with diarrhea in young calves (Mainil 1999). Cattle carry a distinct pattern of Gb3 receptor isoform mixtures in the bovine kidney. This, together with the general absence of receptors on vasculature, could contribute to the apparent resistance of cattle to systemic effects of Stxs. Expression of Gb3 on the bovine intestinal epithelium, together with previously described effects, may affect STEC colonisation in its reservoir hosts and promote the distribution to humans (Hoey et al. 2002). Recently, the prevalence of STEC was studied in twelve Belgian cattle herds of which some animals were diagnosed as STEC-positive at slaughter. Of these 12 herds, nine were confirmed to be culture positive for STEC at farm level, showing a within-herd prevalence of approximately 18.4% (Joris 2012). In three of these herds, a longitudinal study was performed to analyze the persistance and dissemination of STEC. In 43.5 % of the

positive animals, fecal samples were intermittent positive. In 8.7% of the cases, the animals were shedding chronically over a period of at least six to 12 weeks. Furthermore, it was shown that positive animals can shed different strains at different sample points (Joris et al. 2013b). Other studies showed that positive animals became culture negative within two to three months after the first testing (Rahn et al. 1997).

Serum antibodies against O157 lipopolysaccharide (LPS) and Stx1, and -2 frequently occur in bovine samples and in colostrum upon experimental infection (Hoffman et al. 2006). However, these responses are inadequate to clear infections, as they persist over longer periods of time (Stevens et al. 2002c) or do not protect from re-infections. Nevertheless, serum antibodies against EspA and EspB might be a useful tool for screening herds, but not individuals, for O157, O26, and O103 infections (Joris et al. 2013b). *E. coli* O157:H7 prefers the terminal rectum up to the recto-anal junction (RAJ) as primary site for colonization in cattle. This site is characterized by a high density of lymphoid follicles, which could be an important gate towards modulation of the immune system (Naylor et al. 2003).

The shedding of STEC tends to be higher in warmer months, with a peak prevalence in summer and early fall and a drastic decrease in prevalence during the winter months (Hancock et al. 2001). This seasonal effect might be linked to an increased proliferation of the bacteria during warm weather (Hancock et al. 1994), but also the changing day length and corresponding physiological responses within the animal might explain this seasonal patterns (Edrington et al. 2006).

2.7.2 Transmission routes of EHEC to humans

Four principal routes of transmission of EHEC to humans are described: (1) foodborne transmission; (2) waterborne transmission; (3) person-to-person transmission; and (4) direct contact with animals.

Most EHEC outbreaks are associated with foodborne transmissions, as inadequately cooked or baked meat, un- or unsufficiently pasteurized dairy products or outbreaks linked with fermented sausages and apple juice have been reported (Torso et al. 2015; Buvens et al. 2011; Holck et al. 2011; Cody et al. 1999). Worldwide trends towards healthier lifestyle and the ever busier consumer life styles have greatly increased the consumption of ready-to-eat-no-need-to-wash fresh fruits and vegetables.

Unfortunately, fresh produce can become contaminated pre-harvest via fertilizers such as ruminant manure or post-harvest during processing of foods (Feng 2015).

Also water plays an important role in spreading of EHEC since surface waters can become contaminated through draining from pastures or through cow manure-fertilized soil. This source of contamination allows EHEC to spread easily in the environment. A large outbreak in Canada was linked to contamination of the drinking water supply by rainwater run-off contaminated with cattle feces (Hrudey et al. 2003). Also EHEC infections due to contaminated swimming water were reported (Samadpour et al. 2002). Recently, several pathogenic strains were identified in water and fish from pay-to-fish ponds in Brazil. This clearly links proper management of these environments with the risk of foodborne transmissions (Ribeiro et al. 2016).

Person-to-person transmission has been reported in institutional settings such as day-care centres, geriatric homes and hospitals, but also among family members (O'Donnell et al. 2002; Reiss et al. 2006). Since the infectious dose is low (1 to 100 cells) this transmission can easily occur via the fecal-oral route, when hygiene after toilet visits is poor (Welinder-Olsson & Kaijser 2005).

Several EHEC outbreaks have been associated with petting zoos and farms resulting from direct contact with the infected animals followed by inadequate hand washing (Heuvelink et al. 2002; Ihekweazu et al. 2012). Also domestic pets like cats and dogs can be a natural reservoir (Rumi et al. 2012; Morato et al. 2009). In Germany, a 2-year old girl with bloody diarrhea was found to excrete EHEC for three months. Stool samples of her cat yielded the identical EHEC strain (Busch et al. 2007).

2.7.3 EHEC in humans

After ingestion, EHEC passes through the acidic stomach to colonize the gut by adhering to the epithelial cells of the colon, inducing A/E lesions (Lewis et al. 2015). These lesions are characterized by destruction of the microvilli and rearanggement of the cytoskeleton to form pedestals, as described previously in section 2.4. After colonization in the colon, EHEC releases its Stxs, causing microvascular endothial injuries. Subsequently, it is believed that Stx1 passes the epithelial barrier through a transcellular route, while Stx2 uses a paracellular pathway (Hurley et al. 1999). Furthermore, Stxs may also enter the bloodstream by binding and damaging intestinal epithelial cells and

subsequently endothelial cells, causing HC. This is probably an additional way to cross the epithelial barrier (Tarr et al. 2005). In the circulation, the toxins bind to PMN leukocytes and are delivered to endothelial kidney cells (Brigotti et al. 2008). Subsequently, the toxins are released close to endothelial glomerular cells, mesangial and tubular epithelial cells of the kidney and vascular endothelium of the brain. These cells are characterized by a high presence of the Gb3 receptor. Injuries to the vascular endothelium of brain and kidney leads to increased permeability and loss of fluids and erythrocytes, and to thrombosis of small blood vessels (Proulx et al. 2001). The occludation of blood vessels damages erythrocytes, which are removed via the reticuloendothial system leading to hemolytic anemia. Furthermore, HUS is also featured by thrombocytopenia, which might be related to a direct effect of the toxins on platelets or might be related to a toxin-induced endothelial cell injury, exposing the subendothelium. This injury may release prothrombotic factors leading to platelet aggregation, which leads to ischemic damage (Corrigan & Boineau 2001; Proulx et al. 2001).

The clinical outcome of an EHEC infection can range from a symptom-free infection through watery diarrhea, to severe HC and HUS. Characteristically, patients suffer the first three days from watery diarrhea, frequently associated with abdominal cramps, occasionally with nausea and vomiting. In 90% of the cases the diarrhea becomes hemorrhagic within one to three days, starting with a normal platelet count, creatinine concentration and packed-cell volume with no erythrocyte fragmentation (Tarr et al. 2005). Spontaneous recovery after one week occurs in most of the cases. However, the infection can evolve into life-threatening HUS, characterized by acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. Children and elderly have a higher risk of developing HUS (Dundas & Todd 2000). The average mortality rate associated with HUS is 3-17 %, but can increase for elderly people up to 87 % (Griffin & Tauxe 1991). Sometimes not only the kidney and gastrointestinal tract, but also other organs are affected in HUS. The central nervous system was affected in 25 % of the cases and leads to irritability, learning disabilities, lethargy and seizures. More sporadic cases have been linked with cerebral oedema and coma (Amirlak & Amirlak 2006; Elliott & Robins-Browne 2005). In 10 % of the cases, insulin-dependent diabetes mellitus and increase of pancreatic enzymes indicate involvement of the pancreas (Andreoli &

Bergstein 1982). Also a small rate (<1%) have been associated with heart failure and infarction, lung oedema and muscle and skin problems (Siegler 1994).

2.8 Immune responses against EHEC

2.8.1 Immune response and subsequent modulation of host immune system to promote EHEC survival

During intestinal colonization EHEC encounters chemical barriers (e.g. mucins and enzymes in saliva, acidity in the stomach, bile secretion in the small intestine, and antimicrobial proteins throughout the intestine), mechanical barriers (mucus layer) and biological barriers (intestinal microflora) (House et al. 2009; Barnett Foster 2013). When the bacteria have bypassed these barriers, the host immune system is required for control and elimination of pathogens. As a first line of defence, the innate immune system will protect against invading pathogens by activation of pathogen recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs), such as LPS, flagellin, and peptidoglycan (Akira et al. 2006). This recognition results in production of proinflammatory cytokines which stimulate the innate immune response, leading to the development of a more specific and long-term adaptive response to a certain pathogen, mediated by B- and T-cells (O'Neill et al. 2013).

It is unclear to which extent antibodies against *E. coli* O157:H7, produced by the adaptive immune system, are protective against infections since antibodies found in cattle are not capable of preventing re-infections (Joris et al. 2013a; Kieckens et al. 2015). On the other hand in humans, antibodies against common EHEC and EPEC antigens may have a protective effect as they might be the reason for the low prevalence of EHEC infections in areas where EPEC is endemic (Martinez et al. 1999).

The primary site of infection of EHEC and EPEC is the epithelium lining the mucosa of the gastrointestinal tract (Robins-Browne & Hartland 2002). These epithelial cells play a crucial role in homeostatis of the digestive system and coordinate the expression and upregulation of antimicrobial cytokines with a proinflammatory and chemoattractant function, in response to infection. These cytokines include TNF, IL-1,

IL-8, macrophage inflammatory protein 1-alfa (MIP1-α) and, monocyte chemoattractant protein-1 (MCP-1) (Kagnoff & Eckmann 1997).

Different studies have shown that bacterial **flagellin** and **LPS** are important factors affecting proinflammatory cytokine production upon infection (Schüller et al. 2009; Miyamoto et al. 2006; Griener et al. 2011).
Acute inflammation mediates the influx of polymorphonuclear (PMN) leukocytes, mainly neutrophils. These cells are the first leukocytes which are recruited to the site of infection and can kill an invading pathogen by phagocytosis, degranulation or release of neutrophil extracellular traps. Chemoattractive factors, like IL-8 which can be induced by EHEC and EPEC, cause transmigration of neutrophils from the blood into the lamina propria (Hurley et al. 2001). Different infection studies in gnotobiotic piglets show that EHEC and EPEC induce inflammatory cell infiltration in the lamina propria and transmigration of inflammatory cells across the intestinal epithelium into the intestinal lumen (Tzipori et al. 1985; Tzipori et al. 1989). Also patients infected with EHEC show significantly increased numbers of leukocytes in their feces (Slutsker et al. 1997). These studies indicate the importance of neutrophilic migration and subsequent inflammation during EHEC and EPEC infections.

Dahan et al. (2002) have shown in intestinal epithelial cells that the IL-8 production during EHEC or EPEC infection is dependent on a transcription factor in the IL-8 promotor region, AP-1. This transcription factor is activated by MAPK, which are conserved host proteins playing a pivotal role in different cell responses, including regulation of cytokine expression, cytoskeletal reorganization, and stress responses (Davis 1993). IL-8 gene expression in the host is initiated by activation of the key transcriptional regulator of the innate immune system, NF-κB (Mukaida et al. 1990). This transcription factor controls gene expression during inflammation and is activated by various stimuli such as pathogens, stress signals and proinflammatory cytokines like TNF and IL-1β (Figure 1.6) (Li & Verma 2002). When a ligand is recognized by the T-cell receptor, signalling events will be triggered that result in the activation of the IκB kinase (IKK) complex, consisting of IKKβ (IKK2), IKKα (IKK1), and IKKϒ (NEMO). Activated IKK in turn phosphorylates the inhibitory protein of NF-κB (IκB), followed by subsequent ubiquitination (inactivation by attaching ubiquitin) and degradation of IκB by

the host proteasome (Perkins 2007). In a resting cell IκB is bound to p50/p65 dimers, which are released upon proteosomal degradation of IκB and transported through the nuclear pore complex into the nucleus, where they can influence the expression of multiple cytokine genes, including IL-8 (Li & Verma 2002). Upon infection, EHEC is capable of producing different factors that have the potential of upregulating inflammatory cytokine production by intestinal epithelial cells, as a result of activation of MAPK and NF-κB signalling.

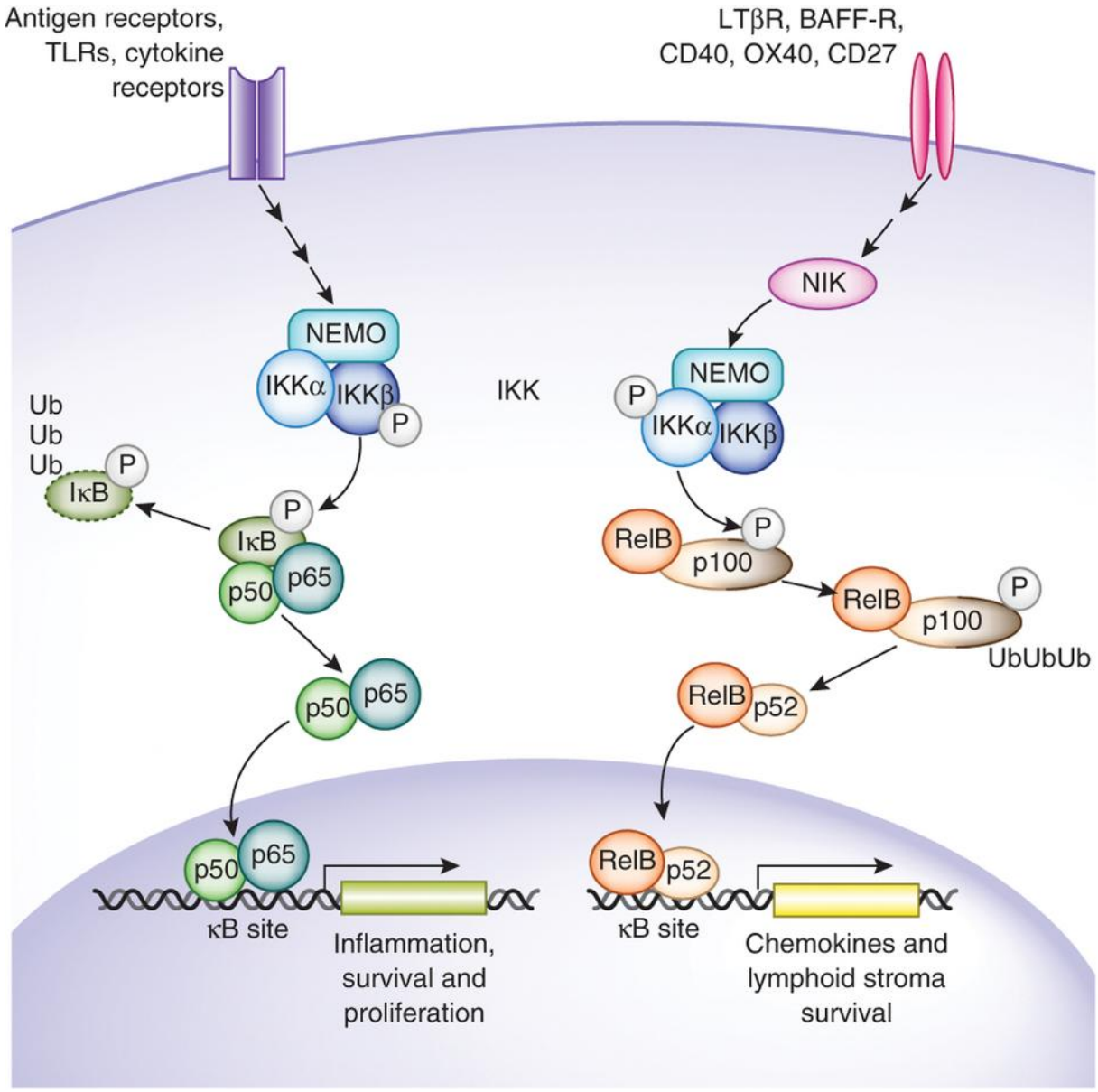

Figure 1.6: Activation of the dormant NF-κB pathway triggered by stimulus-dependent signaling network. The canonical NF-κB pathway (left) induced by signals including antigens, TLR ligands and cytokines such as TNF uses a wide variety of signaling adaptors to engage and activate the IKKβ subunit of the IKK complex. IKKβ phosphorylation of classical IκB proteins bound to NF-κB dimers such as p50-p65 results in ubiquitination (Ub) of IκB and proteasome-induced degradation. This allows NF-κB to enter the nucleus where it binds specific DNA sequences (κB sites) involved in controlling the transcription of genes encoding functions as diverse as inflammation, cell survival and cell division. The noncanonical pathway (right) engaged by members of the TNF-like family of cytokines requires NIK to activate IKKα, which then phosphorylates p100, triggering its proteosomal processing needed for the activation of p52-RelB dimers. Among its functions, this specific NF-κB heterodimer controls gene expression crucial for lymphoid organogenesis. From Gerondakis et al. (2014).

EHEC produce **Stxs** which induce ribotoxic stress responses and apoptosis. Next to receptor mediated uptake of Stx, also endocytosis by macropinocytosis has been described (Malyukova et al. 2009).Increased permeability of the mucosal barrier might allow paracellular Stx transport from the lumen. Once Stx is internalised in epithelial

cells it induces apoptosis, (Smith, 2003) which initiates an inflammatory response and influx of leukocytes. The copious neutrophil migration can enhance Stx translocation from the lumen via enterocytes *in vitro* (Hurley et al. 2001).

Stx alone was also shown to be capable of inducing IL-8 production, related to induction of MAP kinase (MAPK) pathways, JNK and stress-activated protein kinase, and p38, in the intestinal epithelial cells (Smith et al. 2003). Stx-induced increases in cytokine synthesis could be important in augmenting the host mucosal inflammatory response to EHEC infection (Thorpe et al. 1999). Stx could also induce the expression of TNF-α and IL-6 in murine peritoneal macrophages (Tesh et al. 1994). Next to affecting the innate response, Hoffman et al. (2006) demonstrated that Stxs are also capable of modulating the adaptive immune response by delaying the antigen-specific cellular immune response in cattle.

Epithelial cell MAPK and NF-κB pathways (Firgure 1.6) leading to IL-8 secretion were also activated to a significant extent by isolated EHEC **H7 flagellin**, which was active when added to either the apical or basolateral surface of polarized human intestinal epithelial cells (Berin et al. 2002). *In vitro*, it was shown that the most potent activation of inflammation of EHEC/EPEC occurs by Toll like receptor 5 (TLR5) recognition of flagellin, and not Stx signalling through the Gb3 receptor (Miyamoto et al. 2006; Berin et al. 2002). Furthermore, some EHEC strains have shown preference for TLR5 of the follicle-associated epithelium in the gastro-intestinal tract (Schüller et al. 2009). Also Toll-like receptor 4 (TLR4), which binds bacterial LPS, induces the host response resulting in the secretion of proinflammatory cytokines. (Bridger et al. 2010).

Another *E. coli* O157:H7 factor capable of inducing an inflammatory response *in vitro* is the **type IV pilus**, hemorrhagic coli pilus (HCP). Significant increases in the production of IL-8 and TNF, but not IL-2, IL-6, and IL-10, have been observed at the basolateral surface of polarized T84 and HT-29 cells, and in non-polarized HeLa cells during infection with HCP-expressing EHEC strains. These strains were able to induce p38 phosphorylation, and activate ERK1/2 and JNK within three hours after EHEC infection (Ledesma et al. 2010).

2.8.2 Host immune responses: cattle

Unlike humans, cattle can secrete *E. coli* O157:H7 over longer periods of time, although they develop immune responses, without showing clinical signs (Hoffman et al. 2006; Kieckens et al. 2015). The development of immune responses has been a matter of debate for several years, as not all studies showed commensurate results. A study of Johnson et al. (1996) demonstrated that oral inoculation of cattle can induce sustained increases in serum antibodies against O157 LPS and Stx1, but not Stx2 (Johnson et al. 1996). These findings were contradicted by a study of Hoffman et al. (2006), who evidenced that cattle can develop humoral responses against Stx2 and bacterial O157 LPS, but it was shown that only inoculation with $Stx2^-$ and not $Stx2^+$ O157 strains resulted in significant cellular responses. This suggests that Stx-producing *E. coli* O157:H7 can suppress the development of specific cellular immune responses (Hoffman et al. 2006). Another study showed that Stx1 directly affects all main bovine lymphocyte subpopulations by blocking lymphoblast transformation and proliferation in $CD8^+$ T-cell and $CD21^+$ B-cell populations, resulting in a dramatically reduced number of viable $Gb3/CD77^+$ blast cells. This effect is not cytokine-mediated (Menge et al. 2003). A study of Corbishley et al. (2014) observed strain-dependent differences in the kinetics of cellular immune responses against O157:H7. The T-helper cells isolated from the terminal rectum showed an increased expression of interferon-γ (IFN-γ) and T-bet, whereas the $CD4^+$ T-cells, NK- cells and $CD8^+$ and γδT-cells had an increase in proliferation marker Ki67. Next to systemic responses, strong mucosal IgA responses in the bovine terminal rectal mucosa were found after experimental oral inoculation of calves. These responses were directed against multiple antigens including T3SS proteins, O157 LPS, H7 flagellin and OmpC (Nart et al. 2008). Mathematic modelling of mucosal innate immune responses has suggested that 5-9 days post inoculation, the innate immune response nullifies the bacterial replication on the rectal epithelium by either reducing the attachment or increasing the detachment from this epithelium (Tildesley et al. 2012).

2.8.3 Host immune responses: humans

The systemic antibody responses of patients towards EHEC infections have been well investigated, as they might be an important diagnostic marker. Karmali et al. (1983) were the first to suggest that sera from patients suffering from HUS mount an antibody response to Stx, as this serum was able to neutralise Stx. However, later on it was shown

that it was also the case for sera of healthy controls (Barrett et al. 1991). Caprioli et al. (1994) described that the neutralizing activity was not an effect of the immunoglobulin fraction, but was caused by other serum components. Serological enzyme-linked immunosorbent assay's (ELISA's) using the detection of antibodies specific for Stx showed that patients suffering from an EHEC infection, but not all, as well as control individuals can demonstrate a high prevalence of antibodies to Stx (Lingwood et al. 1987; Barrett et al. 1991).

Also serum responses against LPS have been repeatedly reported in patients with HC and HUS (Bitzan et al. 1991; Flores et al. 1997; Jenkins & Chart 1999; Tanaka et al. 2000). IgM and IgA antibodies against LPS were detected in breast milk of an *E. coli* O157-infected pregnant woman (Tanaka et al. 2000) and in saliva of children with HUS (Ludwig et al. 2002). A minority of the HUS patients showed serum responses against enterohaemolysin and H7 flagella (Jenkins & Chart 1999). The responses against LPS might be used for serodiagnosis, but are not protective as recurrent infections with HUS have been described (Siegler et al. 1993).

Next to responses against Stx and LPS, antibodies against intimin, Tir, EspA and EspB have been detected in human sera (Jenkins et al. 2000; Li et al. 2000; Karpman et al. 2002). Li et al. (2000) determined that the responses against the previously mentioned antigens were strongly elevated in patients with HUS, from day 8 after hospitalization onwards. The response against Tir was highest, and maintained high until day 60. However, Karpman et al. (2002) discovered that the response against EspB has a higher diagnostic value, as this response was more significantly associated with an acute response. An important drawback of these responses as diagnostic marker is their presence in healthy control patients due to similarities of EHEC and EPEC antigens (Jenkins et al. 2000; Karpman et al. 2002).

Two studies have tried to estimate the risk factors in HUS patients on cytokine level. A study in children with HUS showed that high IL-8 and low IL-10 are risk factors for developing HUS (Westerholt et al. 2000). In contrast, Murata et al. (1998) showed that increases in IL-6, IL-8, IL-10, TNF-α, and granulocyte colony-stimulating factor levels augmented the risk of HUS development. This differences might be due to differences in the host immune response between an uncomplicated *E. coli* O157:H7

infection and HUS, and is likely an inter-person variability rather than an O157-specific immune response (Proulx et al. 1998).

2.9 Strategies to reduce *E. coli* O157:H7 colonization in ruminants

As we have previously discussed in 2.7.2, transmission routes of *E. coli* O157:H7 to humans can be verydiverse. Most outbreaks are linked to foodborne infections and since ruminants are the most important reservoir for *E. coli* O157:H7, we will focus on strategies to reduce the colonization in these species that will lead to reduction of the prevalence and magnitude of fecal *E. coli* O157 excretion by live cattle (pre-harvest). Pre-harvest intervention methods can be grouped into 3 categories: (1) exposure reduction strategies, (2) exclusion strategies, and (3) direct anti-pathogen strategies (LeJeune & Wetzel 2007).

2.9.1 Reduction of exposure

Exposure reduction involves environmental management targeted at reducing bovine exposure to *E. coli* O157 through biosecurity and environmental niche management such as feed and drinking water hygiene, reduced exposure to insects or wildlife, and improved cleanliness of the bedding or pen floor (LeJeune & Wetzel 2007). The occurrence of the pathogen tended to be higher on hides of dirty looking animals (Nastasijevic et al. 2008). Transfer of bacteria from one animal to another often occurs during transport to slaughterhouses, which leads to contamination of more animals but also to contamination of the transport vehicle (Arthur et al.2007b, Guerini, et al. 2007; Cuesta Alonso et al. 2007). Swabbing of different areas of the hide of cattle on the slaughterline revealed the occurrence of the pathogen: hooves (11.3%), brisket (8.4%), rump (7.0%), neck (4.2) and flank (2.8%). The presence of *E. coli* O157 on cattle hides is a major meat safety risk because, in modern industrial abattoirs where a spillage from guts onto the meat during evisceration occurs only rarely, the key source of microbial contamination of bovine carcass meat is the hide (Nastasijevic et al. 2008). Hide-to-meat microbial cross-contamination during cattle dressing can be via direct contact or via equipment (Tutenel et al. 2003). Good slaughtering practices like cleaning knives and washing hands in between carcasses may prevent contamination, but are time consuming and not always well established. Decontamination measures on carcasses like washing or the use of organic acids are implemented but not always effective (Bosilevac et al. 2006; Arthur et al. 2007a; Penney et al. 2007; Laury et al. 2009). One study highlighted the

importance of correct feed management as the feed itself can be contaminated with *E. coli* O157:H7 (Lynn et al. 1998).

2.9.2 Exclusion strategies

In the category of exclusion strategies, we group dietary modifications such as selection of specific feed components and feeding of probiotics and vaccination in or on exposed animals.

Strategies related to feed management aim at modifying the gastrointestinal flora of ruminants to prevent colonization by or to clear *E. coli* O157:H7 from the gut. They are typically fed high-energy grain diets to increase weight gain and efficiency of feed conversion. Different studies confirmed that the type of feed (grain vs. hay) influences prevalence and shedding in cattle. Barley grain has been positively associated with *E. coli* O157 shedding in both observational and experimental studies (Dargatz et al. 1997; Buchko et al. 2000; Berg et al. 2004). Specifically, Berg et al. (2004) reported that cattle shed a higher concentration of *E. coli* O157 and had higher fecal pH when fed a barley grain diet compared with cattle fed a corn-based diet. The specific mechanism responsible for increased *E. coli* O157 shedding in barley-fed cattle is unknown, but barley has a lower starch content than other traditional cereal grains (Huntington 1997), and is more rapidly and completely digested in the rumen (Orskov 1986; Theurer 1986) which results in less undigested starch for secondary fermentation in the large intestine. This results in an increased pH and decreased volatile fatty acids concentrations in the hindgut and might influence the *E. coli* O157 shedding (Jacob et al. 2009). Dietary changes or withholding feed could induce excretion of *E. coli* O157 in culture negative sheep (Kudva et al. 1995). Also diet switches from grass to alfalfa could reduce the shedding of *E. coli* O157:H7 in sheep. Kudva et al. (1997) hypothesized that a high fiber, low nutrient grass diet decreases volatile fatty acids and increases pH in the sheep gut and is therefore more favourable for *E. coli* O157:H7 then a low fiber, high nutrient alfalfa diet.

Probiotics are live, non-pathogenic micro-organisms that confer a health benefit to the host and have been used in cattle industry for over 20 years to enhance animal health and production (Vande Walle 2010). More recently they have been investigated as a means to reduce foodborne pathogens in animals. *Lactobacillus* strains showed promising results in reducing intestinal colonization and fecal shedding of *E. coli* O157 in cattle (Ohya et al. 2000) and sheep (Lema et al. 2001). Other studies confirmed the potential of

probiotics in reducing EHEC shedding in weaned calves (Tkalcic et al. 2003) and harvest-ready feedlot cattle (Younts-Dahl et al. 2005). More recently, also combined probiotic products have been tested in cattle (Stanford et al. 2014). The beneficial effect of *Lactobacillus* strains might be due to the production of lactic acid (Ogawa et al. 2001), the increase of volatile fatty acid concentrations in feces (Ohya et al. 2000), or the reduction of biofilm formation (Kim et al. 2009).

Different vaccination strategies have been tested as a means to control *E. coli* O157:H7 infection in cattle. Most experimental vaccines are based upon T3SS proteins or H7 antigens and show limited and variable effects. Econiche (developed by Bioniche Life Sciences Inc., Belleville, Ontario, Canada) received full licensing approval from the Canadian Food Inspection Agency in October 2008 and was the first commercially available *E. coli* O157 vaccine for use in cattle. Unfortunately, this vaccine is insufficiently capable of limiting the duration of infection and shedding in cattle, even after three vaccine doses. In 2015, Bioniche sold his animal health branch to the French Vetoquinol and they ceased the production of Econiche, making the product no longer available on the market. Another vaccine strategy is based upon the bacterial needs for iron as a nutrient: a siderophore receptor and porin-protein (SRP) based vaccine formulation disrupts the iron transport system, causing bacterial cell death and has shown to be effective in two cattle feedlot settings (Fox et al. 2009; Thomson et al. 2009). This vaccine received a conditional licence for the U.S. market (Epitopix LCC, Willmar, Minnesota, U.S.) in 2009, so the efficacy still has to be demonstrated with additional experiments.

2.9.3 Direct anti-pathogen strategies

Direct anti-pathogen strategies include treatment with sodium chlorate and bacteriophages, in addition to washing of animals before slaughter. Sodium chlorate can kill *E. coli* O157 but pure cultures gave rise to chlorate-resistance (Callaway et al. 2001). Another study suggested that it is unlikely that chlorate treatment will result in a dissemination of antibiotic resistance, but further research is needed to confirm these results (Callaway et al. 2004). Bacteriophages are viruses that infect bacteria, inject their DNA and make use of the biosynthetic machinery of the bacterial host to produce daughter phages, which are released via host lysis in order to repeat the process in other bacteria (Callaway et al. 2008). They have shown to help the removal of *E. coli* O157:H7

from hard surfaces, fruits, vegetables and ground beef up to 100 % (Abuladze et al. 2008). Although bacteriophages seem very effective, *in vivo* studies in ruminants show very variable results and until today no satisfactory bacteriophage therapy has been reported to eliminate *E. coli* O157:H7 in ruminants. This might be due to the very high specificity of a bacteriophage for a bacterial strain.

Many of the methods mentioned in this topic are promising, but it is expected that interventions should be applied on different levels in the agro-food processing chain, from farms to slaughterhouses, in food industry and distribution and even to the consumer's kitchen. Here, general food safety and hygiene measures for kitchen apply as meat should be always well baked or cooked. Furthermore, the use of untreated dairy products might be avoided, while vegetables should be well washed before eating.

3. Lactoferrin

3.1 Introduction

In 1939, lactoferrin (LF) was first described as an unknown "red fraction" in cow's milk (Sorensen & Sorensen 1939). Over 20 years later, this red protein was defined as a transferrin-like glycoprotein in both human and bovine milk (Groves 1960; Montreuil et al. 1960). The foundations of research for the human variant were put in the late 1970's , when the antimicrobial (Arnold et al. 1977) and immunomodulatory (Broxmeyer et al. 1978) properties were discovered. In the early 1980's, the structure was elucidated (Spik et al. 1982). The possible applications, but also the limited availability of the human variant moved the focus of researchers towards the bovine form. Furthermore, the problems with multiple antimicrobial resistant pathogens and the antibiotics-ban as growth-promoter in Europe, has increased the interest in the protein as well as its availability. Several companies brought human (hLF) and bovine lactoferrin (bLF) on the market as food additive. Since the bovine form is more available and cheaper than the human form, it is more affordable for big scale operations in animal industry as well as in human clinical trials even in developing countries. In this section, an overview is given on the current knowledge of LF, as well as the possible applications against microbial infections.

3.2 Structure of LF

LF is a 78 kDa iron binding glycoprotein that belongs to the transferrin family of non-haem proteins. The amino acid sequence of hLF shows 60% identity with human transferrin (Metz-Boutigue et al. 1984). The three-dimensional structure of LF reveals a single polypeptide chain consisting of an N- and a C-lobe linked by a short α-helix. Each lobe has the same folding and can bind one ferric atom, which is coordinated by four protein ligands: two tyrosines, one histidine, and one aspartate (Anderson et al. 1987). Binding of iron requires a cooperative interaction of both lobes, resulting in an iron-saturated (holo-) and a non-saturated (apo-) form. The iron-saturated LF becomes more "closed" with more resistance against proteolysis whereas the non-saturated form is more "open" and flexible (Baker & Baker 2004) (Figure 1.7). LF displays an unique iron binding structure in the two domains of each lobe that can bind to one Fe^{3+} and one CO_3^{2-} ion. This carbonate ion may play a role in the pH dependent release of iron under low

pH conditions (Baker & Baker 2005; MacGillivray et al. 1998). Improving the passage of LF in the gastric tract, its resistance to trypsin and trypsin-like enzymes is important. However, in the stomach, pepsin is capable of cutting LF into smaller peptides (e.g. lactoferricin), which has a stronger antibacterial effect than the native protein (Tomita et al. 1991).

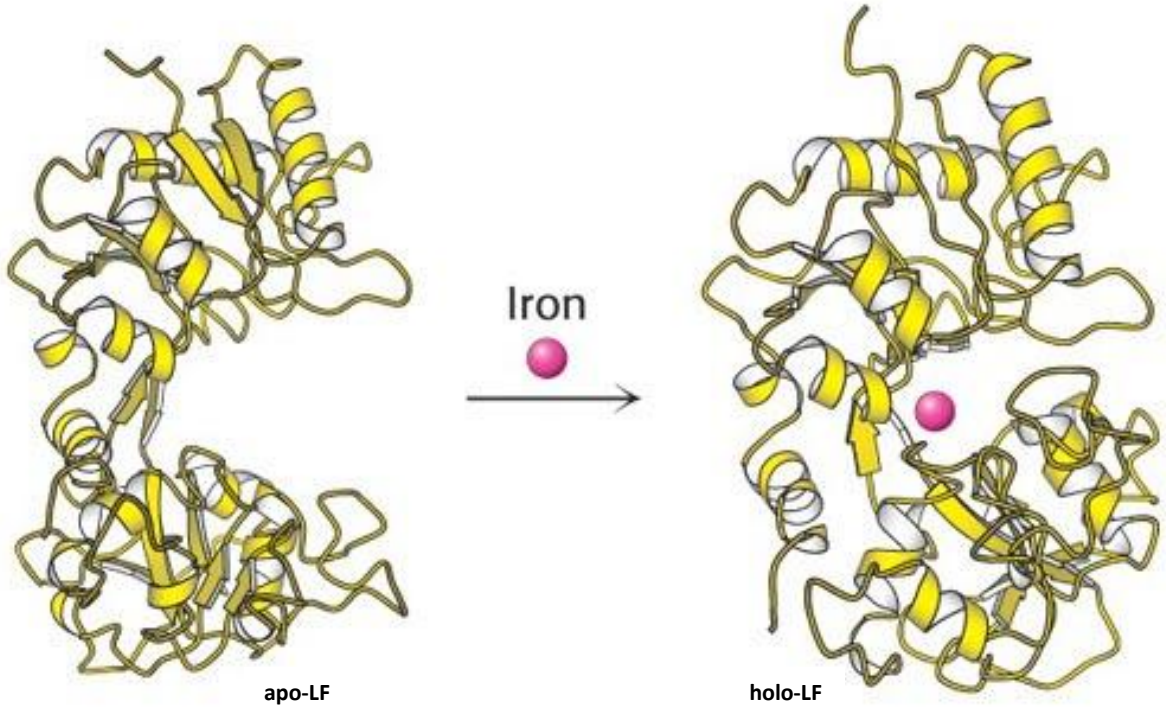

Figure 1.7: Iron binding by LF changes the conformation of LF from the open non-saturated (apo-) form to a more closed iron-saturated conformation (holo-) form (Adapted from: Berg et al. 2002).

3.3 Natural occurrence of LF

LF is found in exocrine secretions of the digestive, respiratory and reproductive system, suggesting a role in the non-specific defence against invading pathogens. An overview of these mucosal secretions and their corresponding LF abundance is given in Table 1.2. The highest concentrations are found in colostrum and milk, making it the second most abundant protein after caseins. Human colostrum contains the highest LF concentration, followed by natural human milk (Masson & Heremans 1971). The concentration of LF is lower in bovine colostrum and milk and depends on the lactation stage, but this milk is available in huge amounts (Cheng et al. 2008). The concentration of LF in blood is low in a healthy situation (1 µg/ml) but can rise up to 200 µg/ml in case of inflammation (Masson & Heremans 1971). This can be explained by the considerable

amounts of LF found in secondary neutrophil granules. Upon microbial infection, neutrophils are rapidly recruited to the site of inflammation, where they play an important role in the innate immune response against bacterial infections (Bennett & Kokocinski 1978).

Table 1.2: Occurence of LF in biological fluids (adapted from: Steijns & van Hooijdonk (2000))

Biological fluid	Amounts reported
Human colostral breast milk	> 7 mg/ml
Human mature breast milk	> 1- 2 mg/ml
Human tear fluid	> 2.2 µg/ml
Human seminal plasma	> 0.4- 1.9 mg/ml
Human synovial fluid	> 10 -80 µg/ml
Human saliva	> 7- 10 µg/ml
Human blood	> 1- 200 µg/ml
Cow's colostral whey	> 1.5 mg/ml
Cow's milk	> 20- 200 µg/ml

3.4 Biological functions and applications of LF

Several biological functions have been awarded to LF and a lot of research is still ongoing. Until now, it is clear that LF plays a role in iron homeostasis (Frazer et al. 2011), cellular growth and differentiation (Bi et al. 1997), host defence against microbial infection (Yekta et al. 2010), protection against viral infections (Wakabayashi et al. 2014), anti-inflammatory activities (Actor et al. 2009) and cancer protection (Zhang et al. 2014). Here, we will focus on the antibacterial effect and its mechanisms of action.

The antibacterial effect of LF has been described *in vitro* and *in vivo* for Gram-positive and Gram-negative bacteria, and influences different levels: it (1) affects iron-uptake; (2) disrupts LPS in the bacterial cell membrane; (3) lyses secreted bacterial proteins; and (4) interacts with bacterial secretion systems.

To maintain iron homeostasis within the cell, bacteria have evolved various types of iron acquisition systems. Ferric iron (Fe^{3+}) is the dominant species in an oxygenated environment, while ferrous iron (Fe^{2+}) is more abundant under anaerobic conditions or at low pH (Lau et al. 2015). LF is an iron-chelator, and can decrease bacterial growth by taking up the ferric iron from the bacterial environment, thereby limiting its use by bacteria at the site of infection. Since LF can bind the ferric atoms with a very high affinity and body secretions and neutrophil granules are mostly iron-free, it can easily

sequester iron and maintain an environment refractory to microbial growth (Aisen & Leibman 1972; Levay & Viljoen 1995; Ward et al. 2005). Next to iron deprivation, LF can also disrupt bacterial LPS which results in an increased membrane permeability and bacterial death (Ellison et al. 1988). A third mechanism used by LF is blocking EPEC-mediated actin polymerization in HEp2 cells and blocking EPEC-induced hemolysis, caused by LF-mediated degradation of secreted proteins necessary for bacterial contact and pore formation, particularly EspB (Ochoa et al. 2003). A fourth mechanism was described by Beeckman et al. (2007) as avian transferrin (ovotransferrin) was capable of inhibiting bacterial irreversible attachment and cell entry by affecting the T3SS of *Chlamydia psittaci* in chicken macrophages.

3.5 Modulation of the immune system by LF

LF was shown to modulate the immune system in two directions: firstly, it can have immunostimulating effects, when the immune response is downregulated as a result of infection; secondly, LF can have immune suppressive effects leading to attenuation of exorbitant immune responses.

LF is an essential element of innate immunity, as it is released from neutrophils within hours after infection. As LF recognizes unique microbial molecules called PAMPs - LPS from the gram-negative cell wall and bacterial unmethylated CpG DNA- it might act as a competitor for these receptors or as a partner molecule, depending on the physiological status of the organism. These immunomodulatory properties are explained by the ability of LF to interact with proteoglycans and receptors on the surface of mammalian cells of the innate (natural killer (NK) cells, neutrophils, macrophages, basophils, neutrophils and mast cells) and adaptive (lymphocytes and antigen-presenting cells (APC's)) immune systems. Through these interactions, LF is able to modulate the migration, maturation and functions of immune cells, and thus to influence both adaptive and innate immune responses (Legrand & Mazurier 2010). The immunomodulation of LF has been extensively studied in *in vitro* and *in vivo* experiments.

LF inhibits LPS signalling by acting as a competitor with serum LPS-binding protein (LBP) and prevents the transfer of LPS to mCD14 bound on the surface of neutrophils (Elass-Rochard et al. 1998). This results in the inhibition of LPS-induced activation and release of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, as described by Miyazawa et al. (1991); Crouch et al. (1992); Mattsby-Baltzer et al. (1996);

and Haversen et al. (2002). LF can also interact indirectly with soluble sCD14 inhibiting the secretion of IL-8 by endothelium (Elass et al. 2002) and interferes with the expression of adhesion molecules on the endothelium which is necessary for recruitment of immune cells to the site of infection (Baveye et al. 2000). The binding with soluble as well as membrane-bound CD14 results in an anti-inflammatory role in the immune system.

It has been shown that LF can modulate the recruitment of immune cells under different circumstances. LF is able to decrease leukocyte infiltration (Yamauchi et al. 2006), diminish recruitment of eosinophils (Kruzel et al. 2006), lessens migration of Langerhans cells (Griffiths et al. 2001), modulates fibroblast motility (Oh et al. 2001) and reduces the neutrophil migration (Dial et al. 2005). The precise mechanisms are not completely understood, but everything seems to result in an attenuation of the immune response.

LF can modulate the phagocytic capacities of cells. However, the mechanism behind is still under debate. Phagocytosis by PMN's is enhanced by the interaction of complement activation products, as complement factor C3. It remains unclear whether this LF activity is related to complement activation since LF was shown either to inhibit (Kijlstra & Jeurissen 1982) or to activate (Rainard 1993) the classical and alternate pathways of complement. More recent research shows that the lactoferricin (LFcin) domain inhibits only the classical complement (Samuelsen et al. 2004). But direct LF binding to PMN's and opsonin-like activity could also be involved (Miyauchi et al. 1998).

Next to affecting the innate immune response, LF also intervenes in adaptive immunity. This connection between innate and adaptive is made by degranulation of LF from neutrophils at inflammation sites. There, LF can promote the activation, maturation and migration of APC's (de la Rosa et al. 2008; Spadaro et al. 2008).

Studies in mice have shown that orally administered LF can influence mucosal and systemic immune responses, as LF can modulate the maturation, differentiation and activation of lymphocytes and promote the antigen-presentation to these cells. It was demonstrated that orally administered bLF strongly elevates the number of $CD3^+$ and $CD4^+$ T-cells (Sfeir et al. 2004) and could even reconstitute a T-cell mediated immune response in immunosuppressed mice (Artym et al. 2003a). LF supports the proliferation and differentiation of Th-cells into the Th1 or the Th2 phenotype, depending on the

host's immune status by affecting the Th1/Th2 cytokine balance. Fischer et al. (2006) have demonstrated the ability of LF to support the proliferation. Thus, LF can cause a Th1 polarization in diseases in which the ability to control infection relies on a strong Th1 response, e.g. an upregulation of Th1 in LF transgenic mice could be associated with clearance of *Streptococcus aureus* (Guillén et al. 2002). However, LF may also reduce the Th1 component to limit excessive inflammatory responses. Besides its effects on cellular immunity, LF can also support the humoral immune response. An increased differentiation of splenic B-cells by LF was described by Zimecki et al. (1995). Furthermore, LF was able to restore the humoral immune response in immunocompromised mice (Artym et al. 2003b). Lastly, LF's binding to CpG-containing oligonucleotides was shown to inhibit their immunostimulatory effects on B-cells (Britigan et al. 2001).

3.6 Effect of LF on *E. coli* O157:H7

In 1998, Shin et al. were the first to report the antibacterial activity of bLF and its peptides, its pepsin hydrolysate bLFH and the active peptide lactoferricin B (LFcinB), against clinical *E. coli* O157:H7 isolates. They showed that LFcinB was the most potent of the three peptides and induced a 4-fold increase of Stx1 and a 4-fold decrease of Stx2 in the supernatant when 5.10^7 CFU/ml were used. If the bacteria were diluted to 10^6 CFU/ml, both Stx1 and 2 dropped below the detection limit. Therefore, it was suggested that high concentrations LFcinB can influence the levels of the extracellular Stxs by interactions with the cell surface and/ or through suppression of bacterial growth (Shin et al. 1998). However, the mechanisms explaining the different effect on Stx1 and Stx2 remain unclear. The effect of LF on the bacterial cell membrane was confirmed as bLfH can cause a collapse of the membrane integrity by pore formation in the inner membrane, leading to the death of the cell (Murdock et al. 2010).

These findings aroused interest for application of LF in food industry and LF and its derivates were confirmed to reduce *E. coli* O157:H7 in ground beef (Venkitanarayanan et al. 1999) and in UHT milk (Murdock & Matthews 2002). The use of Activin™ (activated LF; patented process) was tested in a spray formulation to reduce *E. coli* O157:H7 from beef tissues, and was found to be as effective as the readily established decontamination strategies (Tittor 2003). Furthermore, the applicability of LF in food containing NaCl (Al-Nabulsi & Holley 2006) and in dry sausage production (Al-Nabulsi

& Holley 2007) was confirmed. Next to consumer-ready food applications, the effect of orally administered LF was also confirmed in live hosts. Yekta et al. (2010) described the prevention of persistent colonization of *E. coli* O157:H7 in experimentally infected sheep. The *in vivo* mechanism of action is not completely elucidated, but Blease et al. (2009) suggested the binding of LF and *E. coli* O157:H7 by intelectins. Intelectins are lectins with potential roles in innate immunity, capable of binding bacteria and also function as intestinal receptors for LF. Humans have two intelectins, termed intelectins -1 and -2. Intelectin-1 has been described under different names such as intelectin and intestinal lactoferrin receptor. Intelectin-2 is also known as endothelial lectin HL-2 and is expressed only in the small intestine. Human intelectin-1 is found in a range of tissues, including the small intestine, where it is expressed by Paneth cells, goblet cells and enterocytes. This expression pattern suggests a role for the intelectins as host defence molecules in the small intestine. Also in cattle two intelectins have been described, which seems to have a similar distribution pattern. They both showed only weak expression in jejunum, but were clearly expressed in abomasum and rectum, and bovine intelectin-2 was significantly more expressed in terminal than proximal rectum. Blease et al. (2009) did not find indications for a possible binding of *E. coli* O157:H7 to the bovine intelectins. Yekta et al. (2010) showed that both bLF and hLF can inhibit bacterial growth and attachment to Caco-2 cells, and that the inhibition of attachment was at least partly due to the proteolytic effect of LF on the T3SS proteins. hLF seemed to be more effective, probably due to a more efficient binding of bLF to intelectins (Shin et al. 2008) present on human enterocytes, leading to more uptake and removal of bLF compared to hLF.

Several variables play a role in the efficiency of LF to reduce or inhibit *E. coli* O157:H7 growth. Griffiths et al. (2003) discovered that the iron saturation status of the used LF is an important factor to predict its efficiency, as the vast majority of saturated LF could not significantly reduce the *E. coli* O157:H7 growth. Shimazaki et al. (2000) reported that divalent cations protect bacteria from LF by inducing changes in its tertiary structure yielding a tetrameric form of LF with reduced bio-functionality and simultaneously generating bacterial cell membranes with increased stability. Next to iron saturation and the difference in amounts of cations in the media used, also the purity of the used lactoferrin and the susceptibility of different strains play a role in the outcome of the studies (Al-Nabulsi & Holley 2006).

Chapter II: Aims of the study

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a food-borne pathogen, which causes human illness ranging from self-limited watery or bloody diarrhea and hemorrhagic colitis to acute renal failure and haemolytic uremic syndrome (HUS).

The main reservoirs for *E. coli* O157:H7 are ruminants, mostly cattle. Many studies have investigated the colonization of *E. coli* O157:H7 in cattle. Infection and re-infection appears within the same animal and the infection can persist for long time within cattle herds. Therefore, it is hypothesized that an *E. coli* O157:H7 infection suppresses the immune system in cattle, leading to a reduced and non-protective immune response. The occurrence of serum antibodies against antigenic parts of the bacteria, as well as *E. coli* O157:H7 specific B- and T-cell responses have been described. However, little is known about immune-related gene transcription in cattle upon infection (i) at the site of colonization near the terminal rectum, and (ii) at the gateway to the gut immune system, being the ileal Peyer's patches. The most important colonization site of *E. coli* O157:H7 in cattle is the epithelium of the recto-anal junction, located above the gut-associated lymphoid tissue. Recently, the PhD thesis of Maryam Atef Yekta (2011) and Joanna Rybarczyk (2016) showed promising results of using bovine lactoferrin (bLF), a natural occurring antibacterial and immunomodulating protein, against *E. coli* O157:H7 colonization in sheep and cattle.

Furthermore, the current treatment of EHEC-related diseases in human is limited to supportive care, as antibiotic treatment is contra-indicated because it might increase the frequency of HUS occurrence due to the release of bacterial Stx. Administration of bLF proved to be effective in clearing *E. coli* O157:H7 from the bovine intestine, suggesting that bLF might also be beneficial for future application in EHEC infected humans.

These aspects were addressed and the objectives of this PhD thesis were:

1) Investigating the occurrence of *E. coli* O157:H7 infection induced immuno-suppression in cattle (Chapter III).
2) Evaluating the use of bLF as antibacterial agent against EHEC infection in cattle (Chapter IV).
3) Determining the release of the pro-inflammatory cytokines IL-6 and TNF-α and the chemokine CXCL8 by rectal epithelial cells and tissue explants upon *E. coli* O157:H7 infection in presence or absence of bLF (Chapter V).
4) Exploring the feasibility of using bLF in EHEC infected humans by analyzing the release and biological activity of Shiga toxins upon treatment of different *E. coli* O157:H7 strains with bLF (Chapter VI).

Chapter III: Experimental work

Potential immunosuppressive effects of *Escherichia coli* O157:H7 experimental infection on the bovine host

Kieckens E., Rybarczyk J., Li R.W., Vanrompay D., Cox E.(2016). Potential immunosuppressive effects of Escherichia coli O157:H7 experimental infection on the bovine host. (Manuscript submitted)

3.1 Abstract

Background: Enterohemorrhagic *Escherichia coli* (EHEC), like *E. coli* O157:H7 are frequently detected in bovine fecal samples at slaughter. Cattle do not show clinical symptoms upon infection, but for humans the consequences can be severe after consuming contaminated beef. The immune response against EHEC in cattle cannot always clear the infection as persistent colonization and shedding in infected animals over a period of months often occurs. In previous infection trials using the *E. coli* O157:H7 (Stx^-; NCTC12900) strain, we observed a primary immune response after infection which was unable to protect cattle from re-infection. Furthermore, the duration of the primary infection was mostly shorter (<14 days, n=32) compared to the duration of the secondary infection (>28 days, n=21). These results may reflect a suppression of certain immune pathways, making cattle more prone to persistent colonization after re-infection. To test this, RNA-Sequencing (RNA-Seq) was used for transcriptome analysis of recto-anal junction tissue and ileal Peyer's patches in nine Holstein-Friesian calves in response to a primary and secondary *Escherichia coli* O157:H7 (Stx^-) infection. Non-infected calves served as controls.

Results: In tissues of the recto-anal junction, only 15 genes were found to be significantly affected by a first infection compared to 1159 genes in the ileal Peyer's patches. Re-infection significantly changed the expression of 10 and 17 genes in the recto-anal junction tissue and the Peyer's patches, respectively. After a primary as well as after re-infection, a significant downregulation of 69 immunostimulatory genes and a significant upregulation of seven immune suppressing genes were observed.

Conclusions: Although the recto-anal junction is a major site of colonization by *E. coli* O157:H7 (Stx^-), this area does not seem to be modulated upon infection to the same extent as ileal Peyer's patches as the changes in gene expression were remarkably higher in the ileal Peyer's patches than in the recto-anal junction during a primary but not a secondary infection. We can conclude that the main effect on the transcriptome was immunosuppression by *E. coli* O157:H7 (Stx^-) due to an upregulation of 7 out of 12 genes with immune suppressive effects or a downregulation of 69 out of 94 genes with

immunostimulatory effects . These data might indicate that a primary infection promotes a re-infection with EHEC by suppressing the immune function.

3.2 Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), such as *E. coli* O157:H7, are frequently detected in fecal cattle samples at slaughter (6.3% in Belgium, n = 1281) (Tutenel et al. 2002). Cattle are the main natural reservoir, do not show clinical signs upon infection and can remain asymptomatic carriers for very long periods. If humans become infected by consuming contaminated food, mainly inadequately cooked beef products, the consequences can be severe (Caprioli et al. 2005). After ingestion and subsequent colonization of the human colon, EHEC releases verotoxins causing microvascular endothelial injuries, which might lead to bloody or non-bloody diarrhea, hemorrhagic colitis and the haemolytic uremic syndrome (Vilte et al. 2011; Stevens et al. 2002c).

Recently, the prevalence of EHEC was determined in Belgian cattle herds of which some animals were diagnosed as EHEC-positive at slaughter, showing a within-herd prevalence of approximately 18.4% (Joris 2012). Longitudinal follow-up of herds demonstrated that fecal samples of some animals were intermittently positive, while other animals seemed to have a chronic excretion over a period of at least six to 12 weeks. Furthermore, the shedding patterns showed that positive animals can shed different strains at different sample points (Joris et al. 2013b). Other studies showed that positive animals became culture negative within two to three months after the first testing (Rahn et al. 1997). The immune response of the animals against the EHEC strains, which has been investigated in few studies, might explain these excretion patterns. One study demonstrated that antibodies against the O157 lipopolysaccharide (LPS) and Shiga toxin-1 and -2 (Stx1, Stx2) frequently occur in bovine sera and colostrum upon experimental infection (Hoffman et al. 2006), but this response could not clear the infection, as infected animals secreted bacteria over a period of months (Stevens et al. 2002c). Another study on two farms demonstrated that fecal excretion was not always correlated with *E. coli* secreted protein A (EspA), intimin and, translocated intimin receptor (Tir) specific serum antibody responses. In contrast, 87.5 % of the animals showed a serum antibody response against *Escherichia coli* secreted protein B (EspB) at the same time as their fecal samples

were positive for EHEC O157, O26 or, O111, or 6 weeks after a positive fecal sample. These antibodies persisted, even when shedding had ceased, until the animals were slaughtered, which was 2 to 8 months later, whereas EspA-specific antibodies disappeared within two months. These results indicate that farm animals, which develop an immune response after infection, can become re-infected by different EHEC strains as evidenced by intermittent excretion. In accordance, in our lab we observed in several infection experiments that a primary infection could elicit an antibody response, but this primary immune response was unable to protect against an experimental re-infection with the same strain (*unpublished results*). On the contrary, whereas the primary infection led to fecal shedding during less than two weeks (< 14 days, n=32), a re-infection resulted in excretion for more than four weeks (>28 days, n=21) (Table 3.1).

Table 3.1: Duration of infection after primary or re-infection with *E. coli* O157:H7 (Stx⁻) NCTC12900 strain (*unpublished results*).

Duration of infection	Primary infection (n=32)	Re-infection (n=26)
0-7 days	9	0
7-14 days	23	5
14-21 days	0	4
21-28 days	0	3
> 28 days	0	14

These results may reflect suppression of certain pathways of the immune system by the primary infection, making cattle more prone to persistent colonization by a subsequent infection. In 2003, Naylor et al. (2003) described the preference of *E. coli* O157:H7 for the terminal rectum up to the recto-anal junction (RAJ) as primary site for colonization. This site is characterized by a high density of lymphoid follicles. This predilection for epithelium above mucosa-associated tissue could be important for modulating the immune system. Indeed, *E. coli* O157 is capable of suppressing cell-mediated immune responses in cattle by targeting lymphocytes via their Shiga toxins (Menge et al. 2003; Hoffman et al. 2006), but enterocytes do not have receptors for these toxins, suggesting that close contact with the immune system might be necessary. Furthermore, other bacterial factors might modulate the immune system . The H7 flagellin, bacterial LPS and type IV pilus have been shown to induce proinflammatory responses upon EHEC infection. On the contrary, it has been observed that EHEC as well as EPEC strains could suppress NF-κB and MAPK activation as well as IκB degradation (Hauf & Chakraborty 2003) and could inhibit the production of

proinflammatory cytokines IL-8 andIL-6, early in the infection by different LEE- and non-LEE encoded effectors (Tir, NleB, NleC, NleD, NleE, NleH1, and NleH2) (Stevens & Frankel 2015). Clearance of *E. coli* O157:H7 is associated with an upregulation of Th-1 associated transcripts within the rectal mucosa, the principle site of colonization (Naylor et al. 2003; Corbishley et al. 2014), suggesting that a cellular component of the adaptive immune response may be important in *E. coli* O157:H7 control.

In this study we wanted to obtain insights in genes involved in an immunosuppressive effect of an *E. coli* O157:H7 Stx negative strain. In our experimental infections prolonged excretion was observed after a second infection with this strain. Transcriptome analysis of the ileal Peyer's patches and the RAJ from calves was performed using RNA-seq technology. Samples were obtained from animals that were infected either once or twice or were never in contact with *E. coli* O157:H7.

3.3 Materials and methods

3.3.1 Bacterial strain

The *E. coli* O157:H7 strain NCTC 12900, a well-characterized Shiga-toxin negative EHEC strain of human origin (Dibb-Fuller et al. 2001) was used for experimental infections in calves. We used this verotoxin negative strain for biosafety reasons. Bacteria were grown overnight in Luria Bertani broth (LB, Becton Dickinson, Erembodegem, Belgium) with aeration (200 rpm) at 37°C, harvested by centrifugation (11 337 x g, 5 min), re-suspended in sterile phosphate-buffered saline (PBS, pH 7.4) to a concentration of 10^{10} CFU/10 ml and subsequently used for experimental infections.

3.3.2 Experimental infection of calves and sample collection

Nine 5-week-old Holstein-Friesian calves were randomly assigned to three groups (primary infection, re-infection, and uninfected control; n =3), each reared in separate boxes in isolation units with free access to hay and water (Figure 3.1). These animals were screened to be seronegative for intimin, EspA and EspB, as well negative for fecal shedding of *E. coli* O157:H7. Starting from 8 days of age onwards, the milk diet of the calves was increased and then slowly decreased and completed by a grain-based pellets diet, until the end of the experiment without completely removing milk from the diet.

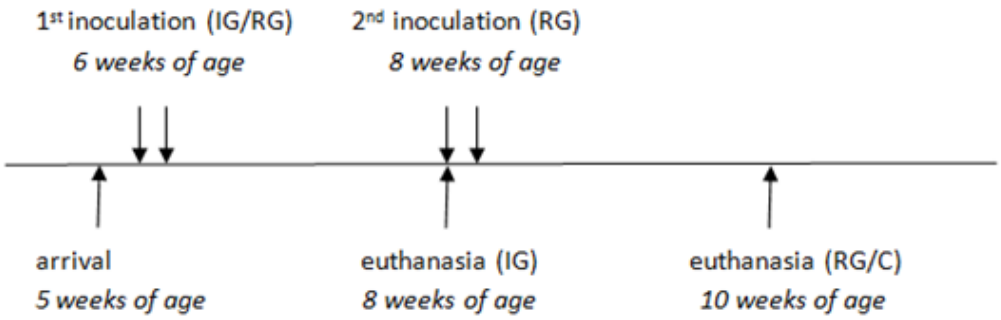

Figure 3.1: Experimental setup.

Six animals were inoculated at the age of six weeks with 10^{10} CFU during two consecutive days as previously described by Kieckens et *al.* (2015). Two weeks after infection, three animals were euthanized (Infection Group, IG). The remaining three infected animals were re-infected and euthanized two weeks later at ten weeks of age (Re-infection Group, RG). At that time, the three uninfected control animals (Control Group, C) of the same breed and age, were also euthanized. Euthanasia was carried out by captive bolt and samples from the ileal Peyers' patches and the RAJ were collected for RNA isolation to analyze the transcriptome profiles by RNA-Seq. Hereto, tissue samples of 1 cm² were cut and rinsed in sterile cold PBS (4°C) and immediately frozen in liquid nitrogen and stored at -80°C until RNA purification. All experimental and animal management procedures were undertaken in accordance to the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2011/082).

3.3.3 Excretion of E. coli O157:H7

Fecal samples were analyzed immediately after sampling, as described by Vande Walle et al. (2011). Briefly, ten gram of faeces was homogenized in 90 ml sterile modified tryptone soy broth (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 20 mg/liter novobiocin (Sigma Aldrich, St. Louis, MO, USA). Enumeration of *E. coli* O157 was performed by plating 10-fold serial dilutions onto MacConkey agar supplemented with sorbitol, cefixime, tellurite, and nalidixic acid (NalCT-SMAC) (Merck, Darmstadt, Germany) and incubating the plates at 37°C for 18 h. The remaining broth was enriched for 6 h at 42°C and subjected to immunomagnetic separation (IMS) using Dynabeads

O157 (Invitrogen, Merelbeke, Belgium), according to the manufacturer's instructions. Finally, 100 µl was plated onto NalCT-SMAC agar and incubated for 18 h at 37°C. Selected sorbitol-negative colonies were confirmed by the O157-specific latex agglutination assay (Oxoid Ltd., Basingstoke, United Kingdom).

3.3.4 RNA extraction and sequencing using RNA-seq

Total RNA was extracted from the tissues using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. Briefly, the samples were grind to a fine powder under liquid nitrogen using a mortar and pestle and homogenized. Then, the lysate was further processed as instructed by Qiagen. The RNA purity was verified using NanoDrop Technology (Thermo Fisher Scientific, Massachusetts, USA) and the RNA concentration was measured. High-quality RNA (260/280 nm ratio ~ 2.0; RNA Rin# > 8.0) was processed using an Illumina TruSeq RNA sample prep kit following manufacturer's instruction (Illumina, San Diego, CA, USA). Individual RNA-Seq libraries were pooled based on their respective sample-specific 6-bp adaptors and sequenced at 50 bp/sequence read using an Illumina HiSeq2000 sequencer.

3.3.5 Data analysis and bioinformatics

The mean number of raw reads generated per sample in the study was 18,910,480.56 $\pm$ 5,610,677.59 (mean $\pm$ SD; n =18). SolexaQA was used for trimming and filtering using default parameters. The resultant reads with < 40 bp in length were discarded. After performing trimming and filtering, the final number of reads for the genome alignment was 16,042,336.72 $\pm$ 5,046,779.84 (mean $\pm$ SD).

The resultant quality reads were aligned to the bovine reference genome (UMD 3.1) using TopHat2 (v2.0.6) (Trapnell et al. 2009) using the following parameters: mismatches allowed: 2 bp; and max insertions: 3 bp and max deletions: 3 bp (using bowtie2 v2.0.2 with INDEL allowed). The SAM output files from the TopHat alignment, along with the GTF file from ENSEMBL bovine genebuild v67.0, were used in the Cuffdiff program in the Cufflink package (v2.0.2) to test for differential gene expression. Mapped reads were normalized based on the upper-quartile normalization method. Cuffdiff models the variance in fragment counts across replicates using the negative binomial distribution (Anders & Huber 2010).

Results were considered significant for $p < 0.05$. Differentially expressed genes identified in the transcriptome were further analyzed using GeneOntology (GO) analysis (https://github.com/tanghaibao/goatools). A Fisher's exact test was used for enrichment of certain GO terms. A multiple correction control (permutation to control false discovery rate, FDR) was implemented to set up the threshold to obtain the list of significantly over-represented GO-terms as previously described (Li & Schroeder 2012).

Ingenuity Pathway Analysis (IPA pathways) and Path designer was used to visualize connections between differentially expressed genes.

The fold change was reported in the result section for every gene in brackets.

3.4 Results

3.4.1 Excretion of E. coli O157:H7(Stx⁻)

Average excretion patterns of the infected animals per group are shown in Figure 3.2. All animals were negative before infection and the highest peak of bacteria in faeces was detected at day 4 post infection. Within 14 days after the primary infection, all animals of the primary infection group became negative. The second infection gave rise to a lower level of bacterial shedding and the animals were still positive at the timepoint of euthanasia.

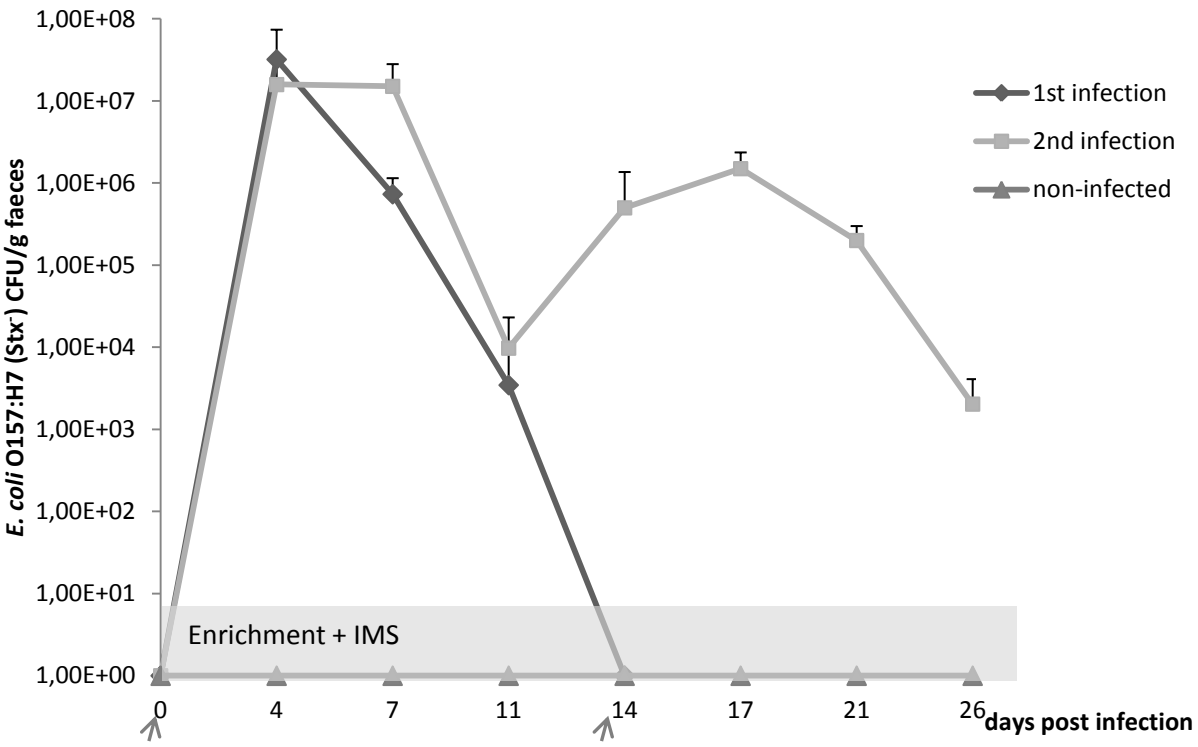

Figure 3.2: Average faecal excretion of infected animals. Error bars indicate standard deviations. Arrows on the x-axis indicate the timepoint of first and second infection.

3.4.2 Transcriptome analysis of gut tissues

A total of 21,046 genes were detected at least once in one of the nine RAJ samples or in one of the nine ileal Peyer's patches samples. The number of genes expressed per sample was 18,753.22 $\pm$ 276.94 (Mean $\pm$ SD). The number of genes with mean hits $\geq$ 5.0 was 16,788 whereas the core transcriptome of the RAJ and the ileal Peyer's patches consisted of 16,948 and 17,197 genes, respectively. This core transcriptome incorporates genes represented by at least one sequence hit in each of the samples tested.

We used the transcript abundance classification as described by Li et al. (2012). Assuming 300,000 mRNA molecules per cell, approximately 70.68% of genes transcribed in the RAJ and 70.55% of genes transcribed in the ileal Peyer's patches can be classified into "very rare" with a relative abundance of $\leq$15 molecules per cell (Table 3.2), followed by "rare" (16-99 molecules per cell) at 16.18% and 17.29%, respectively. "Not expressed" genes (0 molecules per cell) were calculated as 11.17% and 10.18%, whereas "moderately abundant" genes (100-500 molecules per cell) accounted for 1.74% and 1.78%. "Abundant" genes accounted for only 0.23% and 0.21% part of the transcriptome.

Table 3.2: Transcript abundance in the recto-anal junction and Ileal Peyer's patches.

Transcript category	Recto-anal junction	Ileal Peyer's patches
Not expressed	11.17%	10.18%
Very rare	70.68%	70.55%
Rare	16.18%	17.29%
Moderately abundant	1.74%	1.78%
Abundant	0.23%	0.21%

3.4.3 Genes significantly influenced by E. coli O157:H7 experimental infections

In the RAJ, the primary site of *E. coli* O157:H7 colonization in cattle, fifteen genes were found to be significantly affected by a primary infection with *E. coli* O157:H7 (Stx^-) whereas ten genes were affected after re-infection compared to the uninfected control group (false discovery rate, FDR < 0.1). Only one gene (*FABP2*) appeared to be significantly impacted by both primary infection and re-infection with *E. coli* O157:H7 (Stx^-) (Figure 3.3). Three out of fifteen genes that were significantly affected during the primary infection could be linked to an immune function (*KLRJ1, MARCO, CCL20*); one was upregulated and two downregulated (Table 3.3). In the ileal Peyer's patches 1159 genes were significantly influenced by a primary infection compared to the control group and only seventeen genes were significantly affected by the re-infection compared to the same control, indicating a larger effect on the transcriptome during primary infection compared to a re-infection. Seven genes were significantly different regulated after a primary as well as after a re-infection (Figure 3.4). The function of 103 out of 1159 genes that were differently regulated during the primary infection could be traced back to the immune system.

Table 3.3: Overview of numbers of up- and downregulated genes in respect to their effect on the immune system.

	Immune stimulating effect		Immune suppressive effect	
	# upregulated	# downregulated	# upregulated	# downregulated
Recto-anal junction	/	2	1	/
Ileum + PP	25	67	6	5

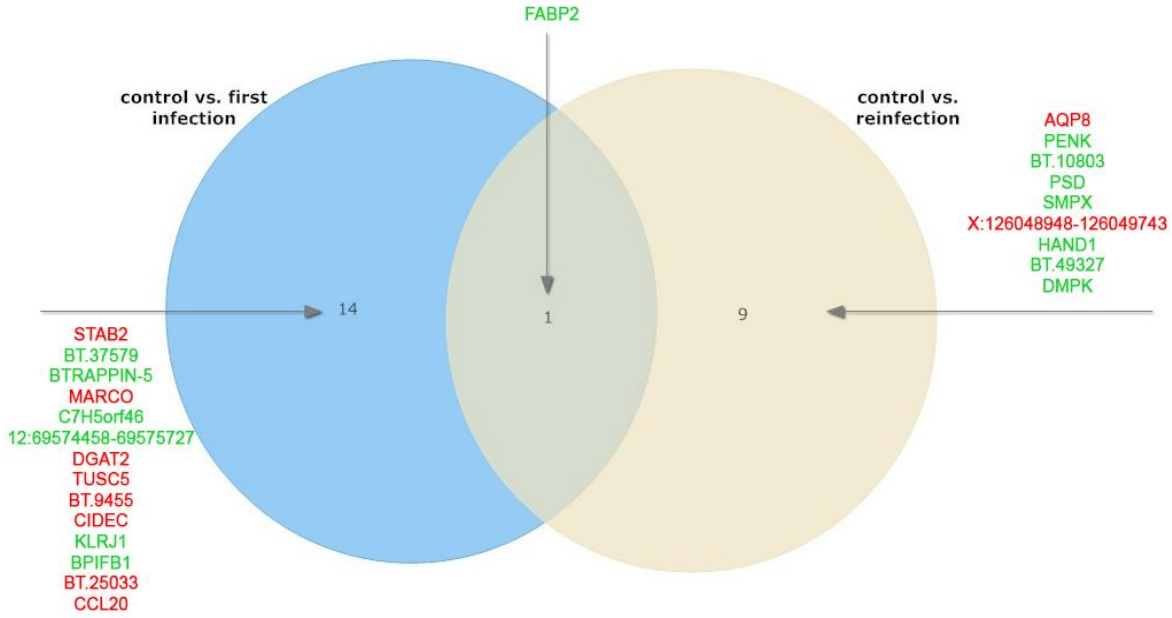

Figure 3.3: Venn diagram of differentially expressed genes in the recto-anal junction. Upregulated genes are indicated in green, while downregulated genes are indicated in red.

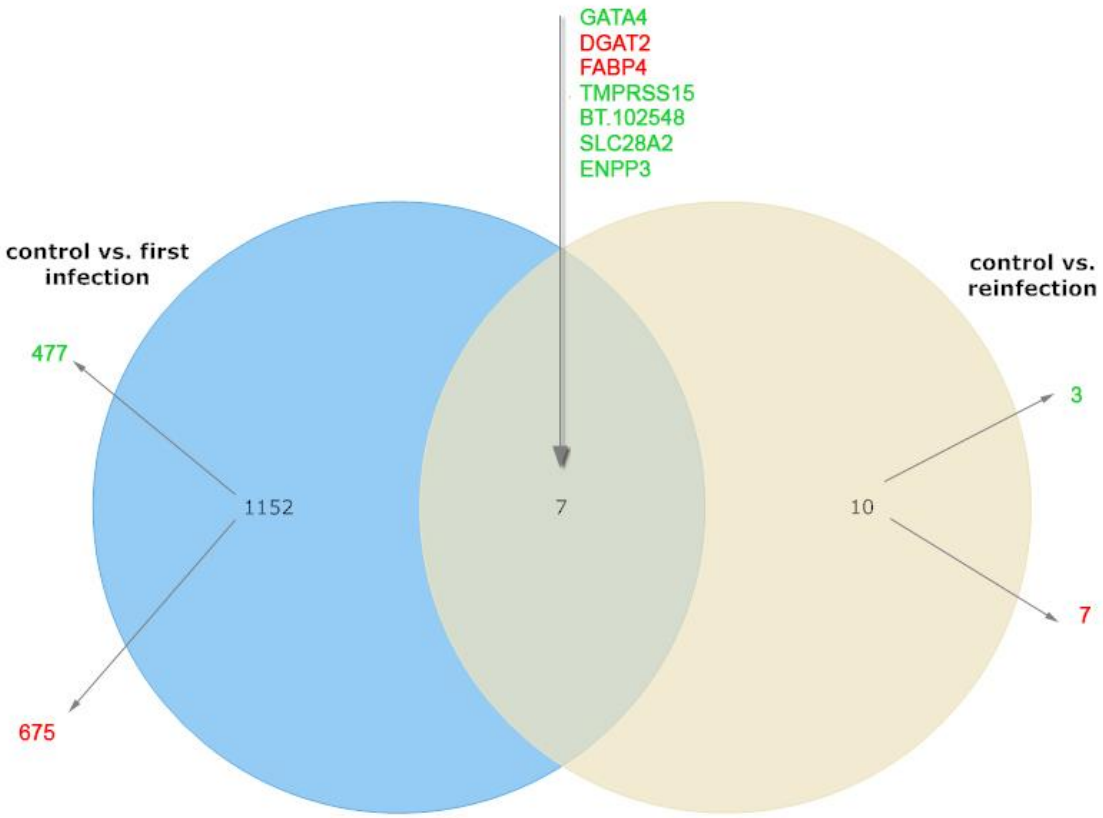

Figure 3.4: Venn diagram of differentially expressed genes in ileal Peyer's patches. Upregulated genes are indicated in green, while downregulated genes are indicated in red.

3.4.4 Gene ontology (GO) analysis

A total of 15 genes were significantly affected by a primary infection with *E. coli* O157:H7 (Stx^-) in the RAJ at a cutoff FDR <10%. Enrichment with Bonferroni corrected P value = 0.0115 reveals three genes possessing scavenger receptor activity (GO:0005044) and three genes having cargo receptor activity (GO:0038024). In the Peyer's patches ileum, 1159 genes were significantly affected by a primary *E. coli* O157:H7 (Stx^-)infection. After enrichment, we could identify 997 genes linked to a molecular function (GO:0003674), 882 genes to biological processes (GO:0008150), 673 genes were linked to a cellular process (GO:0009987) and 561 genes to a single-organism process (GO:0044699). Also GO-terms for metabolic process (GO:0008152), single-organism cellular process (GO:0044763), catalytic activity (GO:0003824), response to stimulus (GO:0050896), cellular response to stimulus (GO:0051716), nitrogen compound metabolic process (GO:0006807), single-organism metabolic process (GO:0044710), hydrolase activity (GO:0016787), small molecule metabolic process (GO:0044281), and

response to stress (GO:0006950) were found for 492, 488, 405, 329, 247, 230, 223, 204, 145 and 123 genes, respectively, out of 1159 genes that were significantly impacted. GO-terms for <100 genes were not reported in this manuscript.

3.4.5 Regulatory gene networks

The IPA software was used to further examine the RNA-Seq dataset. Uploading the dataset for the RAJ after a primary infection, the database could assign two relationships between the significantly regulated genes, whereas for the re-infection four relationships could be detected in both cases linked to antimicrobial responses. When the data for ileal Peyer's patches was uploaded, IPA could identify three different networks that might play a role in *E. coli* O157:H7 (Stx^-) infection, related to antimicrobial response (28 relationships) (Figure 3.5), inflammatory response (69 relationships) (Figure 3.6) and infectious disease (166 relationships) (Figure 3.7).

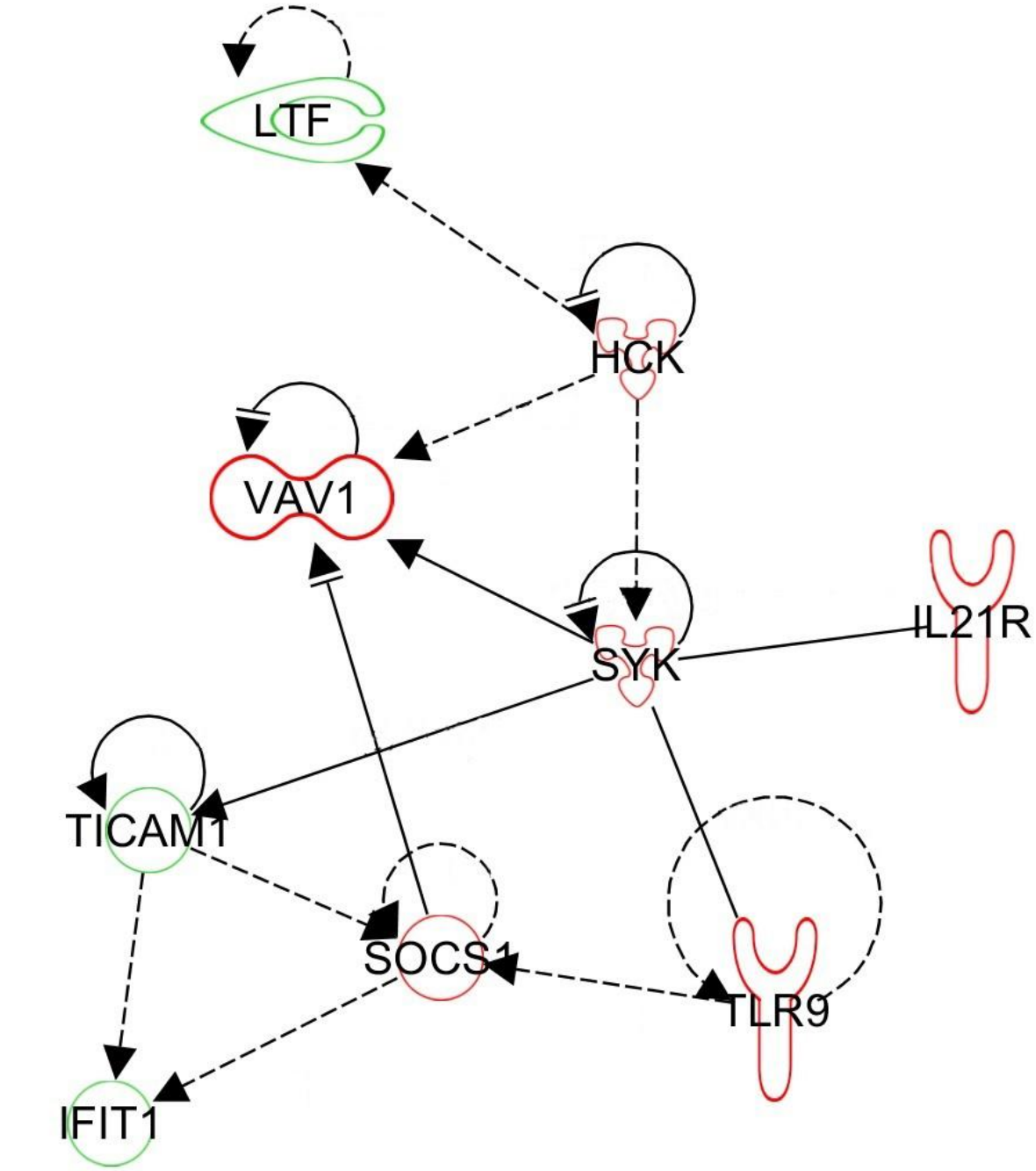

Figure 3.5: Regulatory network related to antimicrobial response impacted in the ileal Peyer's patches of calves after a primary infection with *E. coli* O157:H7 (Stx$^-$). Up- and downregulation is represented by green and red colours, respectively.

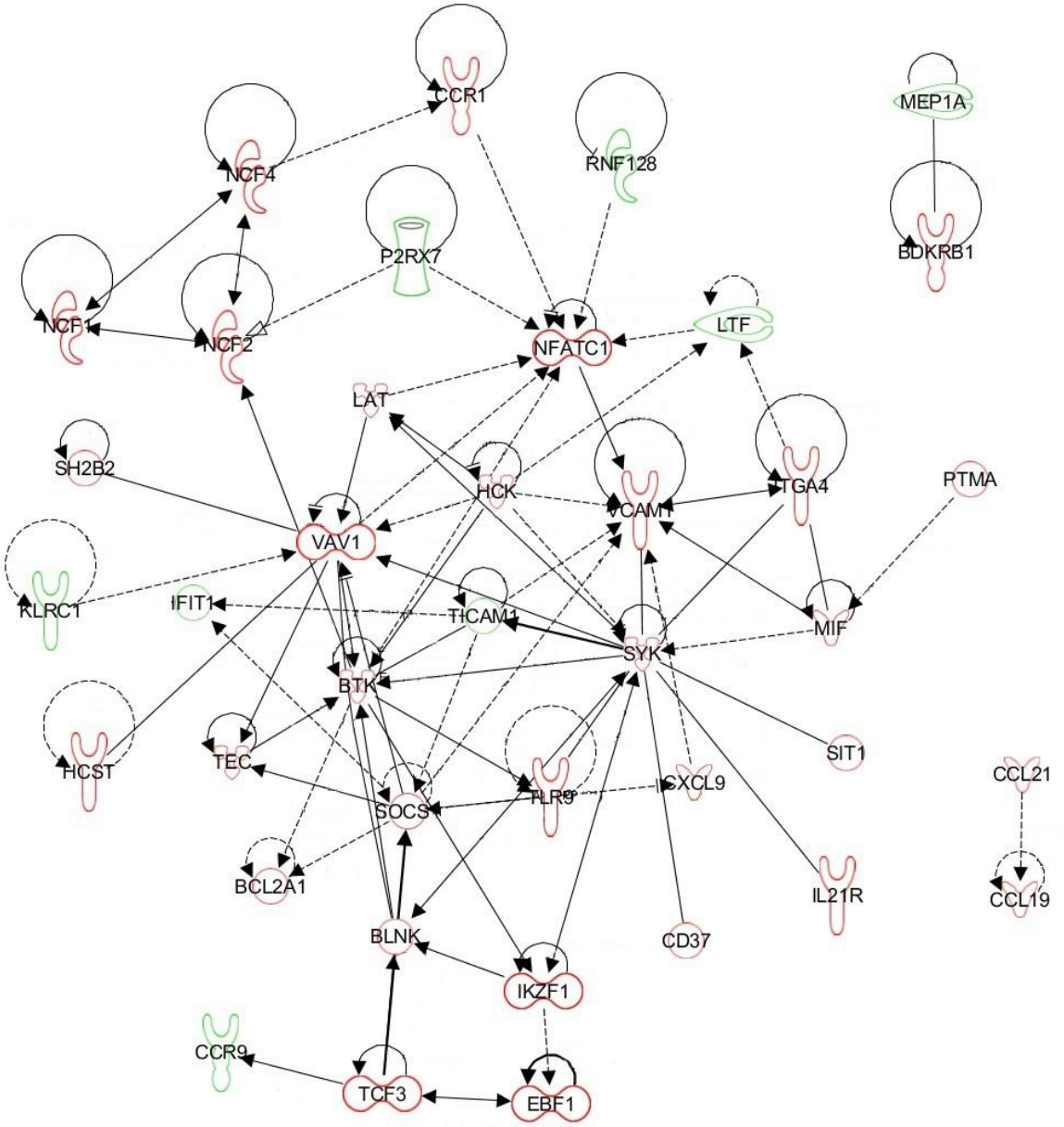

Figure 3.6: Regulatory network related to inflammatory response impacted in the ileal Peyer's patches of calves after a primary infection with *E. coli* O157:H7 (Stx$^-$). Up- and downregulation is represented by green and red colours, respectively.

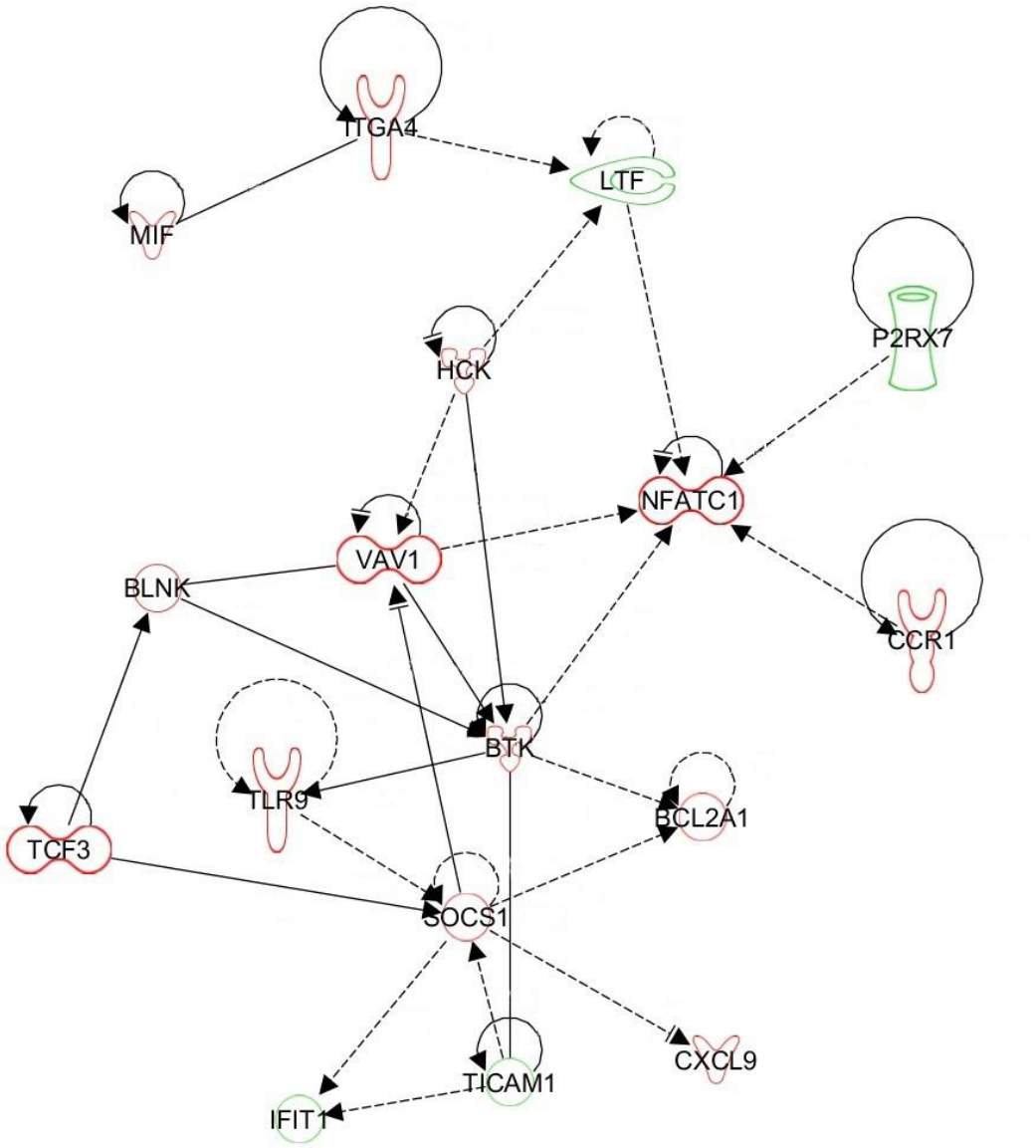

Figure 3.7: Regulatory network related to infectious disease impacted in the ileal Peyer's patches of calves after a primary infection with *E. coli* O157:H7 (Stx$^-$). Up- and downregulation is represented by green and red colours, respectively.

3.4.6 Possible effect of E. coli O157:H7 experimental infection on the function of immune cells

In order to gain insight in the impact of the differential mRNA expression, the data were arranged according to their effect on different immune cells in the specific tissues: lymphocytes (3.4.5.1), natural killer cells (3.4.5.2), monocytes and macrophages (3.4.5.3), dendritic cells (3.4.5.4), granulocytes (3.4.5.5). Only genes with the highest fold changes per cell type are reported, whereas the tables give an overview of the fold

changes (log_2) >2 in both RAJ and ileal Peyer's patches. The gene expression results for RAJ and ileal Peyer's patches after primary infection are represented in Table 3.4 and 3.5, respectively.

Table 3.4: Gene expression results for recto-anal junction samples with Fold change (log_2) >2.

Gene name*	Gene function	Fold change (log_2)	Reference
KLRJ1 +(Ly49)	Natural killer cell receptor binding host MHC I as a mechanism of self/health recognition. Binding of the ligand results in an inhibitory signal to prevent killing of the target cell.	--3.90	Storset et al. (2003)
MARCO$^-$	Innate immune defense. Can bind Gram-negative bacteria to stimulate clearing of the pathogen.	4.88	(Elomaa et al. 1998)
CCL20$^-$	Strongly chemotactic for lymphocytes and weakly for neutrophils.	3.73	(Mohammed et al. 2007)

(*: $^+$ = upregulated; $^-$ = downregulated).

Table 3.5: Gene expression results for Ileal Peyer's patches samples with Fold change (log_2) >2.

Gene Name*	Gene function	Fold change (log_2)	Reference
ENPP7$^+$	Might have an inflammatory effect, as it could degrade and inactivate platelet-activating factor (PAF).	-9.93	(Wu et al. 2006)
PDIA2$^+$	Helps to load antigenic peptides into MHC I molecules and is therefore important in antigen recognition and clearing.	-4.41	(LeBrasseur 2006)
BT.36112$^+$	(KIR) Killer cell immunoglobulin receptor suppresses the cytotoxic activity of NK cells.	--2.93	(Vilches & Parham 2002)
MGC137099$^+$	Is preferentially expressed on Th2 cells and is together with SEMA4A a stimulatory molecule for T-cell activation.	-2.54	(European Bioinformatics Institute 2015)
PRLR$^+$	Cytokine receptor and important in the JAK-STAT, JAK-RUSH, Ras-Raf-MAPK and PI-3K pathways.	-2.47	(Bouchard et al. 1999), (Lee et al. 1999), (Amaral et al. 2004)
LY6G6E$^+$	Possible role of Ly-6 family members in T-cell activation, differentiation, and maturation (mouse studies)	-2.42	(Mallya et al. 2006)
BTRAPPIN-5$^+$	Multifunctional host-defense peptide with anti-proteolytic, anti-inflammatory and anti-microbial activities.	-2.25	(Kato et al. 2010)

Gene Name*	Gene function	Fold change (log_2)	Reference
SUSD2$^+$	Contributes to evasion of immune responses by induction of apoptosis in activated T-cells	-2.25	(Watson et al. 2011)
KLRJ1$^+$	(see Table 3 on RAJ)	-2.05	(Storset et al. 2003)
FCRLA$^-$	Leading to inflammatory responses and antibody-mediated cellular cytotoxicity.	7.21	(Inozume et al. 2007)
CXCL13$^-$	Chemokine B-lymphocyte chemoattractant.	6.64	(Legler et al. 1998)
DEFB5$^-$	Bovine neutrophil β-defensins exert broad spectrum of antimicrobial activities against several species that cause mastitis as *S. aureus, E. coli, K. pneumoniae,* and *P. aeruginosa*	6.45	(Alnakip et al. 2014)
BT.53744$^-$	Development and differentiation of B-cells into plasma cells.	6.01	(International Molecular Exchange Consortium 2015)
TNFRSF13C$^-$	Mature B-cell survival.	5.99	(Thompson et al. 2001)
CD79B$^-$	Initiation of the signal transduction cascade activated by the B-cell antigen receptor complex which will lead to antigen presentation.	5.81	(Luisiri et al. 1996), (Tseng et al. 1997), (Pelanda et al. 2002)
SRCRB4D$^-$	Regulation of innate and adaptive immune responses.	5.45	(Online Mendelian Inheritance in Man 2004)
CD180$^-$	Controls B-cell recognition and signaling of LPS.	4.60	(NCBI Reference Sequence Database 2008)
FCRL1$^-$	Functions in B-cell activation and differentiation.	4.43	(Gauld et al. 2002), (Harwood & Batista 2010)
CLEC4E$^-$	Induces secretion of inflammatory cytokines after binding of ligands (such as damaged cells, funghi and microbacteria).	4.40	(Miyake et al. 2010)
CXCR5$^-$	Chemokine plays an essential role in B-cell migration.	4.00	(Sáez de Guinoa et al. 2011)
CD19$^-$	Acts as a B-cell coreceptor in conjunction with CD21 and CD81.	3.84	(van Zelm et al. 2006)
P2RY8$^-$	Regulator of the immune response.	3.81	(Amisten et al. 2007)
LTA$^-$	Mediates a large variety of inflammatory, immunostimulatory and antiviral responses.	3.712	(NCBI Reference Sequence Database 2012)

Gene Name*	Gene function	Fold change (log_2)	Reference
CXCR4-	Receptor for SDF-1, has potent chemotactic activity for lymphocytes.	3.67	(Tamamis & Floudas 2014)
TLR10-	Role in pathogen recognition and activation of innate immunity.	3.64	(Lee et al. 2014)
SPP1-	Chemotactic for many cell types including macrophages, dendritic cells, and T cells; it enhances B lymphocyte immunoglobulin production and proliferation. In inflammatory situations it stimulates both pro- and anti-inflammatory processes.	3.50	(Wang & Denhardt 2008)
BANK1-	Is expressed during development of B-lineage cells.	3.34	(Dymecki et al. 1992)
FCRL3-	Regulator of the immune system.	3.32	(Swainson et al. 2010)
LTB-	LTs are important for innate and adaptive immune responses by controlling the expression of several adhesion molecules, other cytokines and chemokines	3.03	(Creus et al. 2012)
DOK3-	Negative regulator of JNK signaling in B-cells.	3.03	(Robson et al. 2004)
KLRF1-	Activating lectin-like receptor expressed on NK-cells and stimulates their cytotoxicity and cytokine release.	3.00	(Kuttruff et al. 2009)
FCER2-	Transportation in antibody feedback regulation	2.85	(Kijimoto-Ochiai 2002)
FCAMR-	is expressed constitutively on the majority of B-lymphocytes and macrophages; FCAMR functions as a receptor for the Fc fragment of IgA and IgM and binds IgA and IgM with high affinity and mediates their endocytosis	2.82	(Shibuya et al. 2000), (McDonald et al. 2002)
CCL19-	Antimicrobial gene; may play a role in normal lymphocyte recirculation and homing. It also plays an important role in trafficking of T cells in thymus, and in T cell and B cell migration to secondary lymphoid organs	2.54	(National Center for Biotechnology Information Gene 2014)
TNFSF8-	Involved in cell differentiation, apoptosis and immune response	2.43	(Wu et al. 2011)
SOCS1-	Negative regulator of cytokine signaling.	2.334	(Krebs & Hilton 2001)
TIMD4-	Enhances the engulfment of apoptotic cells: involved in regulating T-cell proliferation and lymphotoxin signaling.	2.30	(UniProt 2015)
SIT1-	Negatively regulates T-cell receptor mediated signaling in T-cells.	2.28	(Marie-Cardine et al. 1999)

Gene Name*	Gene function	Fold change (log_2)	Reference
BDKRB1$^-$	Receptor binding leads to increase in the cytosolic calcium ion concentration, resulting in chronic and acute inflammatory responses.	2.26	(Talbot et al. 2012), (Enquist et al. 2014)
AKAP5$^-$	Is expressed in T-lymphocytes and may function to inhibit IL-2; IL-2 is part of the body's natural responses to microbial infections.	2.21	(Schillace et al. 2002)
PGLYRP2$^-$	Recognizes peptidoglycan, a component of bacterial cell walls.	2.20	(Dziarski & Gupta 2010)
CD37$^-$	T-cell and B-cell interactions.	2.13	(Knobeloch et al. 2000)

(*: $^+$ = upregulated; $^-$ = downregulated).

3.4.6.1 Lymphocytes

In the RAJ, a significant downregulation of *Chemokine C-C motif ligand 20 (CCL20)* (fold change (log_2) = 3,74) was observed, which is strongly chemotactic for immature dendritic cells, and B- and T-lymphocytes (Mohammed et al. 2007).

Furthermore, in the ileal Peyer's patches, *Sushi domain containing 2 (SUSD2)* was significantly upregulated (fold change (log_2) = -2,25). *SUSD2* can interact with *Galectin-1* which is known to contribute to the evasion of immune responses of tumors and infectious organisms by inducing apoptosis of activated T cells (Watson et al. 2011). The activation of T-lymphocytes might be inhibited by the significant downregulation of the expression of *T cell immunoglobulin and mucin domain 4 (TIMD4)* (fold change (log_2) =2.30), responsible for regulation of Th1 responses (Meyers et al. 2005). *C-X-C motif ligand 13 chemokine (CXCL13)*, strongly expressed in the follicles of the spleen, lymph nodes, and Peyer's patches promoting the migration of B-lymphocytes in the lymph nodes (Sáez de Guinoa et al. 2011) was significantly downregulated in the ileum (fold change (log_2) =6.64). *Interleukin 17 receptor E-like (IL17REL)*, a member of the *Interleukin 17 (IL17)* cytokine receptor family that functions as a receptor for the proinflammatory cytokine responding to invading extracellular pathogens (Wu et al. 2011) was found to be significantly downregulated (fold change (log_2) =1.18) upon *E. coli* O157:H7 infection. A significant downregulation of *prothymosin alpha (PTMA)*, a tumor necrosis factor receptor (*RELT*), *Interleukin-21 receptor (IL21R)*, a guanine nucleotide exchange factor (*VAV1*) with log_2(fold change) = 1.29, 1.39, 1.48, 1.52 respectively, and many more immune response

stimulating genes linked with lymphocyte responses were seen in the ileal Peyer's patches.

3.4.6.2 Natural Killer cells

Effector functions of NK cells are controlled by a balance of inhibitory and stimulatory signals. In the RAJ, a strong significant upregulation of *killer cell lectin-like receptor (KLRJ1)* was observed (fold change(log_2) = -3.90). *KLRJ1* is probably important for the NK cell recognition of target cells, which are certain tumor cells, virally infected cells and host MHC class I cells as a mechanism of self/health recognition. An upregulation of *KLRJ1* would imply an upregulation of the inhibitory signal, causing more survival of the target cells (Storset et al. 2003). In the ileal Peyer's patches, a significant upregulation of *protein disulfide isomerase (PDIA2)* (fold change (log_2) = -4.41), *killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail 1 (KIR2DS1)* (fold change (log_2) = -1.89), *killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail 5A (KIR2DL5A)* (fold change (log_2) = -1.48) and *killer cell lectin-like receptor subfamily C, member 1-like (KLRC1)* (fold change (log_2) = -1.36) was observed. In contrast, a significant downregulation of *IL21R* (log_2(fold change) = 1.44) important for the proliferation and differentiation of B-, T- and NK-cells, *leukocyte-associated immunoglobulin-like receptor 1 (LAIR1)* (fold change (log_2) = 0.52) an inhibitory receptor found on NK cells, T cells, and B cells regulating the immune response to prevent lysis of cells recognized as self (Poggi & Zocchi 2014), *hematopoietic cell signal transducer (HCST)* (fold change (log_2) = 1.35) playing a role in cell survival and proliferation by activation of NK and T cell responses (André et al. 2004; Karimi et al. 2005) and *killer cell lectin-like receptor subfamily F, member 1 (KLRF1)* (fold change (log_2) = 3.43) stimulating the cytotoxicity and cytokine release of NK cells (Kuttruff et al. 2009), was observed.

3.4.6.3 Monocytes and macrophages

In the RAJ, *macrophage receptor with collagenous structure (MARCO)* was significantly downregulated (fold change (log_2) = 4.88). This is a receptor which is part of the innate antimicrobial immune system binding both Gram-negative and Gram-positive bacteria

via an extracellular, C-terminal, scavenger receptor cysteine-rich (*SRCR*) domain (Elomaa et al. 1998).

At the ileal Peyer's patches significant upregulation was seen of *ectonucleotide pyrophosphatase/phosphodiesterase 7 (ENPP7)*, *PDIA2*, *carboxypeptidase M (CPM)* important for monocyte to macrophage differentiation (Rehli et al. 2002), the inflammatory *purinergic receptor ligand-gated ion channel, 7 (P2RX7)* and *egf-like module containing, mucin-like, hormone receptor-like 4 pseudogene (EMR4P)*, a member of the *EGF-TM7* receptor gene family which is thought to be important for adhesion and migration of macrophages (McKnight & Gordon 1998) with (fold changes (log_2) of -9.93, -4.41, -0.78, -0.64, and -0.14, respectively. These genes result in stimulation of the immune system on level of monocytes and macrophages. On the other hand, *macrophage migration inhibitory factor (MIF)* which is important for the acute immune response (Rex et al. 2014) was significantly downregulated as well as *chemokine C-C motif receptor 1 (CCR1)* playing a role in recruitment of leukocytes to the effector site (Anderson et al. 2010) (fold change (log_2) = 1.17 and 1.46, respectively). Furthermore, *sialic acid binding Ig-like lectin 10 (SIGLEC10)* which is a negative regulator of immune signaling by functioning as an inhibitory receptor (Whitney et al. 2001) and *B cell linker (BLNK)*, an adaptor molecule linked to the pathway activated by B-cell antigen receptor signals (NaserEddin et al. 2015), were significantly downregulated, with (fold changes (log_2) of 6.53 and 1.94, respectively.

3.4.6.4 Dendritic cells (DC's)

No differential mRNA expression of genes indicating an effect on DC's was seen in the RAJ. In samples from the ileal Peyer's patches, a significant upregulation of *PDIA2*, *toll-like receptor adaptor molecule 1 (TICAM1)* (fold change (log_2)= -4.41) and *EMR4P* was observed, whereas *SIGLEC10* and *MIF* were significantly downregulated.

3.4.5.5 Granulocytes

In the RAJ, there was no differential regulation observed that could have a direct influence on granulocytes. However, in the ileal Peyer's patches, a significant upregulation of *ENPP7*, *PDIA2*, *chemokine C-C motif ligand 24 (CCL24)* (fold change (log_2) = -1.93), which is chemotactic for eosinophils and neutrophils (White et al. 1997) and of

EMR4P was detected. While a significant downregulation of *neutrophil cytosolic factor 1 (NCF1)* (fold change (log_2) = 1.71), *neutrophil cytosolic factor 2 (NCF2)* (fold change (log_2) =1.70) and *neutrophil cytosolic factor 4 (NCF4)* (fold change (log_2) = 1.22) important for the formation of the neutrophil phagosome leading to phagocytosis of bacteria (Nordenfelt & Tapper 2001), *CCR1*, *hemopoietic cell kinase (HCK)* (fold change (log_2) = 1.70) playing a role in the neutrophil migration and degranulation (Quintrell et al. 1987), *SIGLEC10* and *vascular cell adhesion molecule 1 (VCAM1)* (fold change (log_2) = 2.79) mediating the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium (Hwang et al. 1997), was noticed.

3.5 Discussion

It is well known that some pathogens have developed mechanisms to prolong their persistence in a host and can modulate the host immune response in different ways in order to increase their survival in the host (Janeway et al. 2005). This might be done by passive evasion of the immune surveillance for instance by altering expressed antigens, as *Streptococcus pneumonia* does. Another way to survive is actively modulating and interfering with networks that are part of the immune defence. This is done when EHEC and EPEC inject modulatory proteins into the host cell using T3SS (Schmid-Hempel 2009; Croxen & Finlay 2010). Furthermore, the suppression of immune responses via TLR4 by uropathogenic *E. coli* (UPEC) leads to a decrease of IL-6 and IL-8 release (Hunstad & Justice 2010). To our knowledge, this is the first RNA-Seq study highlighting the effect of a primary infection and a re-infection with *E. coli* O157:H7 in cattle, leading to a better understanding of the transient and sometimes recurrent pattern of EHEC infections. We could identify an important difference in regulation of the transcriptome after an initial contact with the bacteria: the ileal Peyer's patches were more influenced by the infection compared to the RAJ, which is part of the primary site for colonization in cattle (Naylor et al. 2003). In this study we were able to show suppression of immunity on different levels of the innate and adaptive immune response, indicating that *E. coli* O157:H7 can modulate this response in a wide variety of ways. This discussion focuses on genes that were significantly affected in our study and which were previously linked with bacterial infections in other studies.

Our data suggests that an infection with *E. coli* O157:H7 is capable of modulating the immune response causing dramatic decreases in *CCL20*. A similar trend was observed in a bacterial infection with *Mycoplasma gallisepticum* in chicken, where a downregulation in mRNA expression of *CCL20* as well as of *IL-1*, *IL-8* and *IL-12p40* genes was seen. These results indicate the importance of lymphocyte and monocyte chemotactic factors in development of disease, but also the fast occurrence of modulations of the host immune responses by bacteria (Mohammed et al. 2007).

Granulysin delivers granzymes into bacteria to kill diverse bacterial strains. In *Escherichia coli*, granzymes cleave electron transport chain complex I and oxidative stress defense proteins, generating reactive oxygen species (ROS) that rapidly kill bacteria (Walch et al. 2014). An upregulation of granulysin is part of the adaptive immune response against bacterial infections.

A study of St John et al. (2009) demonstrated downregulation of *CXCL13* and *CCL21* during an infection of draining lymph nodes by *Salmonella typhimurium*. The pathogen disrupts the lymph node architecture and cellular trafficking, which enhances its virulence and could serve as a mechanism of immune suppression used by pathogens that primarily target lymphoid tissue (St John & Abraham 2009). In our study, we could observe a significant downregulation of *CXCL13* but not of *CCL21* altough the bacterium is in close contact to lymphoid dense tissue in the RAJ and the ileum.

We observed a downregulation of *IL17REL*, a gene which is important for a fast inflammatory response. The cells of the innate immune system are the first line of defense against pathogen and their cytokines govern the differentiation of T- helper cells. Their pattern-recognition receptors, which are not specific for any particular epitope, allow them to respond to a wide variety of microbial invaders by producing cytokines that activate T-cells of the adaptive immune system. T-helper 17 cells produce IL17 and this is particularly important for immunity at epithelial and mucosal surfaces, as indicated by the pattern of expression of their chemokine receptors and effector cytokines. Several pathogens, like gram-positive *Propionibacterium acnes* and Gram-negative *Citrobacter rodentium*, induce mainly Th17 responses (Miossec et al. 2009). Furthermore, Luo et al.(2015) have shown that F4 fimbriae of ETEC can elicit an IL-17 response in piglets, suggesting a role in protection of the host against ETEC infection.

A downregulation of *IL21R* might play a role in the persistence of colonization during the primary EHEC-infection and re-infection of the host, since the IL-21-IL-21R pathway is important in the development of immune responses, as abnormal signaling through the IL-21R/γc/JAK3/STAT3 pathway leads to defective humoral immune responses to both T-dependent and T-independent antigens and impairs the establishment of long-lasting B-cell memory (Desjardins & Mazer 2013). A bacterial infection can elicit IgM memory B-cells which requires T cell dependent and IL-21R signaling. The study of Yates et al. (2013) demonstrates that T cell-dependent IgM memory B cells can be elicited at high frequency and can play an important role in maintaining long-term immunity during bacterial infection.

In the RAJ, we could observe the downregulation of *MARCO*, a receptor that can bind Gram-negative bacteria and is only found on macrophages of the marginal zone of the spleen and lymph nodes (Elomaa et al. 1998). Pinheiro da Silva et al. (2007) have shown that *E. coli* are capable of hijacking inhibitory ITAMs leading to a decrease in MARCO-mediated phagocytosis (Pinheiro da Silva et al. 2008). This observation is indicating a decrease in antibacterial protection of the host at the primary site of EHEC infection.

A limitation of our study is that most information from gene function databases is derived from studies that are not performed in cattle but in human and mice, therefore we have to take into consideration species-specific differences which we unfortunately cannot control. This study is important since it is the first study using the highly-accurate and sensitive RNA-Seq technique to study the effect of EHEC on the cattle immune responses. These insights are crucial to better understand EHEC colonization and shedding within herds and our data could contribute to effective measures to control EHEC colonization in ruminants, thereby reducing zoonotic food-borne infections in humans.

3.7 Conclusions

We can conclude that the main effect on the transcriptome was immune suppression by *E. coli* O157:H7 due to an upregulation of 7 out of 12 genes with immune suppressive effects or a downregulation of 69 out of 94 genes with immunostimulatory effects (Table 3.3). Furthermore, the changes in gene expression were remarkably higher

in the ileal Peyer's patches (1159 genes) than in the RAJ (15 genes) during a primary infection. This effect was less obvious after the re-infection (17 and 10 genes, respectively). The data might indicate that a primary infection promotes a re-infection with EHEC by suppressing the immune function.

Chapter IV: Experimental work

Modulation of systemic immune responses in *Escherichia coli* O157:H7-infected calves by bovine Lactoferrin

Adapted from: Kieckens E., Rybarczyk J., De Zutter L., Duchateau D., Vanrompay D., Cox E.(2015). Clearance of *Escherichia coli* O157:H7 infection in calves by rectal administration of bovine Lactoferrin. Applied and Environmental Microbiology 81(5): 1644-1651.

4.1 Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) strains, of which *E. coli* O157:H7 is the best-studied serotype, are an important group of foodborne pathogens causing severe illness in humans worldwide. The main reservoirs for EHEC are ruminants, mostly cattle, which harbor the bacteria in their intestinal tracts without showing clinical symptoms. Many studies have shown that EHEC uses a wide range of factors to suppress host immune responses thereby promoting persistent infection. In this study, we used bovine lactoferrin (bLF), a naturally occurring immunomodulating and bactericidal protein, as a possible stimulator of the systemic host immune response against *E. coli* O157:H7 infection in cattle. Nine 3-month-old Holstein-Friesian calves were experimentally infected with *E. coli* O157:H7 (Stx^-; strain NCTC12900). Three animals received a daily rectal spray treatment with bLF, three animals received an oral treatment, and three animals served as a control group. Blood samples were collected weekly and fecal samples twice weekly to monitor antibody responses and fecal excretion, respectively. All groups developed serum responses, but no clear differences could be observed between the groups although animals in the rectal group ceased shedding within 26 days of the experimental treatment and remained negative, whereas no beneficial effect of bLF on bacterial shedding was observed in the oral group. Animals of the oral and control group remained positive until the time of euthanasia (day 61). The results indicate that the use of bLF as a rectal treatment can be a useful strategy to preclude further transmission of EHEC infections from cattle to humans. The mechanism behind this effect might be, at least partly, due to stimulation of the local immune responses by bLF, as no systemic effect was observed.

4.2. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a foodborne pathogen which causes human illness ranging from self-limited watery or bloody diarrhea and hemorrhagic colitis to acute renal failure and hemolytic uremic syndrome (Sheng et al. 2006). Infection in humans is typically acquired through the ingestion of contaminated food or water, direct contact with infected animals, or person-to-person transmission. The main reservoirs for *E. coli* O157:H7 are ruminants, mostly cattle, which, in contrast to humans, harbor the bacteria in the gastrointestinal tract without showing apparent illness (Hancock et al. 1997; Chapman et al. 1993). The persistence of *E. coli* O157:H7

and the duration of shedding in naturally and experimentally infected cattle are highly variable and vary from days to months (Hancock et al. 1997; Shere et al. 2002; Cobbaut et al. 2009). Mechanisms leading to persistence of *E. coli* O157:H7 in ruminants are not fully understood, impeding the search for a successful approach to combat *E. coli* O157:H7 zoonotic transfer (Yekta 2011). Nevertheless, many studies have shown the importance of effector molecules produced by EHEC, which influence the host immune response in order to promote persistent colonization (reviewed in Chapter I, section 2.6).

The current treatment of EHEC-related diseases in humans is limited to supportive care, as antibiotic treatment is contraindicated because it might increase the frequency of hemolytic uremic syndrome occurrence due to the release of bacterial Shiga toxins (Grif et al. 1998). Different strategies to reduce the EHEC carriage rates in ruminants, including vaccination (Rabinovitz et al. 2012), probiotic treatment (Ohya et al. 2000), bacteriophages (Sheng et al. 2006), and modification of diet (Callaway et al. 2011), have been tested. Unfortunately, these strategies show only limited results. Currently, no methods that can substantially reduce EHEC carriage rates in ruminants are available. Thus, innovative strategies which could effectively reduce *E. coli* O157:H7 carriage in ruminants are urgently required for diminishing the risk to public health.

Emerging multiple-antimicrobial-resistant pathogens and the full ban on in-feed antibiotics in Europe have increased research on natural antimicrobial proteins such as transferrins. Lactoferrin, a member of the transferrin protein family, is an iron-binding glycoprotein that is found in many exocrine secretions, including milk, tears, saliva, and serum. Lactoferrin exhibits antibacterial activity against Gram-negative and Gram-positive bacteria in a variety of ways. First, it sequesters iron, which is an essential growth factor for microorganisms (Otto et al. 1992). Second, lactoferrin is capable of destabilizing the outer membranes of Gram-negative bacteria, which results in the release of bacterial lipopolysaccharides (LPS) from the bacterial membrane (Ellison et al. 1988). Another important feature of lactoferrin is its immune-modulating effect (reviewed in Chapter I, Section 3.5). It can influence both innate and adaptive immune responses and seems to activate immune responses when suppressed or attenuate responses when too high. In this study, we investigated if bovine lactoferrin (bLF) can stimulate the systemic immune response upon *E. coli* O157:H7 colonization in cattle.

Several studies have identified the epithelium of the recto-anal junction, located above the gut-associated lymphoid tissue, as the major colonization site of EHEC in cattle (Naylor et al. 2003; Grauke et al. 2002). The present study evaluated the curative effect of orally or rectally administered bLF in an experimental *E. coli* O157:H7 infection model in calves, which we established in our laboratory (*unpublished results, 2013*). Three major questions were addressed. (i) Can orally administered bLF sufficiently reach the predilection site for *E. coli* O157:H7, the rectal mucosa, to stimulate the immune response and contribute to clearance of *E. coli* O157:H7 colonization? (ii) Does rectal administration of bLF result in stimulation of immune responses which lead to *E. coli* O157:H7 clearance? (iii) Does bLF treatment influence the systemic antibody response against *E. coli* O157:H7 virulence proteins, and might these responses be used in the future to monitor the infection status of a cattle herd?

4.3. Materials and methods

4.3.1. EHEC strain

E. coli O157:H7 strain NCTC12900, a well-characterized Shiga toxin-negative EHEC strain of human origin with nalidixic acid resistance (Dibb-Fuller et al. 2001), was used for experimental infections in calves. We used this Shiga toxin-negative strain for biosafety reasons. Bacteria were grown overnight in Luria-Bertani broth (LB) with aeration (200 rpm) at 37°C, harvested by centrifugation (11,337 x g, 5 min), resuspended in sterile phosphate-buffered saline (PBS), to a concentration of 10^{10} CFU/10 ml, and subsequently used for experimental infections.

4.3.2. Bovine lactoferrin

bLF with 92% purity and 16% iron saturation (manufacturer [Ingredia Nutritional, France] information), derived from bovine milk, was used in this study. Using a bovine Ig enzyme-linked immunosorbent assay (ELISA), the absence of bovine antibodies (AbD Serotec) in the bLF was confirmed.

4.3.3. Experimental infection of calves and sampling

Nine three-month-old calves (Holstein-Friesian; Hindryckx N.V., Ichtegem) were randomly assigned to three groups (oral, rectal, and control) of three animals, each reared in a separate pen. The calves were fed grain-based pellets and were allowed free access to

hay and water. All animals were screened to be seronegative for intimin, EspA, and EspB and the feces of these animals were free of *E. coli* O157:H7 as demonstrated by direct plating and isolation (the procedure is described below). Experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC2012/170).

The experimental infection was carried out one week after arrival of the animals. First, using a nursing bottle, calves were given 300 ml of a 10% NaHCO3 solution in order to close the esophageal groove and to allow the bacterial inoculum to drain directly into the abomasum. Each animal was infected with 10^{10} CFU *E. coli* O157:H7 (Stx^-) for two consecutive days (days 0 and 1 of the experiment) and re-infected on days 7 and 8. Treatment with bLF started on day 11 and continued until day 61 for the oral and control groups and until day 43 for the rectal group. The oral group received bLF orally (1.5 g twice a day) suspended in a volume of 10 ml sodium bicarbonate buffer (10%). The rectal group was treated with bLF powder, which was dispersed by air blast onto the rectum (300 mg per day) using a 20-cm-long polyvinyl chloride tube (diameter, 1 cm). The control group received only the bicarbonate buffer orally.

Serum samples (taken from the jugular vein) were collected to detect intimin-, EspA-, EspB- and -specific antibody titers before the primary infection on day 0, before re-infection on day 7, and subsequently on a weekly basis for the duration of the experiment. In all groups, fecal excretion of *E. coli* O157:H7 was monitored twice a week from day 1 onwards until *E. coli* O157:H7 was no longer present in the feces. Animals in the rectal group were euthanized at day 43, while animals in the oral and the control groups were euthanized at day 61. At euthanasia, tissues and contents of the jejunum, ileum, cecum, colon, and rectum were sampled for enumeration of the inoculated strain.

4.3.4. Serum antibody response against virulence factors of E. coli O157:H7

Blood samples were processed directly after collection. Briefly, sera were heat inactivated (30 min at 56°C) and kaolin treated. Polysorb 96-well plates (Polysorb Immuno Plates; Nunc, Roskilde, Denmark) were coated (200 ng/well) with recombinant intimin, EspA, or EspB in phosphate buffered saline (PBS) and incubated overnight at 4°C. Nonspecific binding sites were blocked for 1 h at 37°C by adding PBS plus 0.2% Tween 80. After washing with PBS plus 0.2% Tween 20, the plates were incubated with 2-fold dilution series of serum in PBS plus 0.05% Tween 20, followed by washing and incubating (1 h at 37°C) with affinity chromatography-purified horseradish peroxidise (HRP)-conjugated sheep anti-cattle IgA and IgG antibodies (AbD Serotec, United Kingdom). After a final washing step, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) diammonium salt (ABTS) (Roche Diagnostics, Vilvoorde, Belgium) was added. The optical density at 405 nm (OD_{405}) was measured. Positive- and negative-control sera were kindly provided by the Laboratory of Veterinary Public Health and Food Safety (Merelbeke) (Joris 2012). The cut-off value was determined as mean of the value for the negative-control sera $\pm$ three times the standard deviation (SD). For the calculation of the titers, the maximum dilution that exceeded the cut-off value was used. This values were subsequently log2 transformed.

4.3.5. Excretion and intestinal presence of E. coli O157:H7(Stx$^-$)

Fecal samples were analyzed immediately after sampling, as described by Vande Walle et al. (2011). Briefly, 10 g of feces was homogenized in 90 ml sterile modified tryptone soy broth (mTSB, Oxoid Ltd., Hanst, United Kingdom) supplemented with 20 mg/liter novobiocin (Sigma Aldrich, St. Louis, MO, USA). Enumeration of *E. coli* O157 (Stx$^-$) was performed by plating 10-fold serial dilutions onto MacConkey agar supplemented with sorbitol, cefixime, tellurite, and nalidixic acid (NalCT-SMAC) (Merck, Darmstadt, Germany) and incubating the plates at 37°C for 18 h. The remaining broth was enriched for 6 h at 42°C and subjected to immunomagnetic separation (IMS) using Dynabeads (Invitrogen, Merelbeke, Belgium), according to the manufacturer's instructions. Finally, 100 µl was plated onto NalCT-SMAC agar and incubated for 18 h at 37°C. Selected sorbitol-negative colonies were confirmed by the O157-specific latex agglutination assay (Oxoid Ltd., Basingstoke, United Kingdom).

At euthanasia, 10 g of content from the jejunum, ileum, cecum, colon, and rectum and 10 g of tissues (washed with sterile PBS) from the jejunum with and without Peyer's patches (PP), ileum with and without PP, cecum, colon, and recto-anal junction were tested for the presence of *E. coli* O157:H7 by direct plate counts, as described for the fecal samples.

4.3.6. Statistical analysis

Statistical analysis was performed based on the linear fixed-effects model with treatment as the categorical fixed effect and the area under a curve (AUC) as the response variable. The data represent mean $\pm$ standard deviation. A P-value of <0.025 (Bonferroni adjusted) was considered statistically significant. Colony counts were log10 transformed for data analysis. If *E. coli* O157:H7 could not be detected by direct plating but only by enrichment, a concentration of 10 CFU/g was assigned to the sample (Vande Walle et al. 2011).

4.4. Results

4.4.1 Serum antibody responses against virulence factors of E. coli O157:H7

IgG responses against intimin, EspB, and EspA are presented in Figure 4.1 and IgA responses in Figure 4.2. The responses in all 3 groups showed some similarities, such as (i) the occurrence of low IgG titers against all 3 virulence factors, (ii) the appearance of an IgG response against EspB at 14 days post-primary infection (dppi) until 38 to 43 dppi, (iii) an EspB-specific IgA response which peaked at 14 dppi, and (iv) an intimin-specific IgA response which peaked 33 dppi. The EspA-specific antibody responses were different between groups. EspA-specific IgG was lowest and shortest in duration in the control group (21 dppi), slightly higher en clearly longer in the rectal group (7, 14, 21, and 33 dppi), and the oral group (14, 21, 27, 33 dppi), with in the latter group a second increase 61 dppi. The opposite was seen for the EspA-specific IgA response, which longest in duration in the control group (21, 27, and 33 dppi) and very short in the rectal group (21 dppi) to absent in the oral group. The intimin-specific IgG response resembled the EspB response in the control group and the oral group but not in the rectal group, where intimin specific IgG was detected at 14 dppi only, whereas the intimin-specific IgA response was most pronounced in the rectal group and lower and of shorter duration in both other groups. Another important difference was the reappearance of an IgG response against all three type III secretion system (TTSS) proteins at the end of the

experiment (61 dppi) in the oral group and not in control group, as if a re-infection occurred in the oral group. Animals of the rectal group were already euthanized at this timepoint. Furthermore, EspB-specific IgA appeared and disappeared 3 times in the oral group during the observation period but was absent at the end of the experiment.

Comparing the rectal group with both other groups showed a tendency for a less pronounced intimin-specific IgG response and an enhanced intimin-specific IgA response, even though not significant.

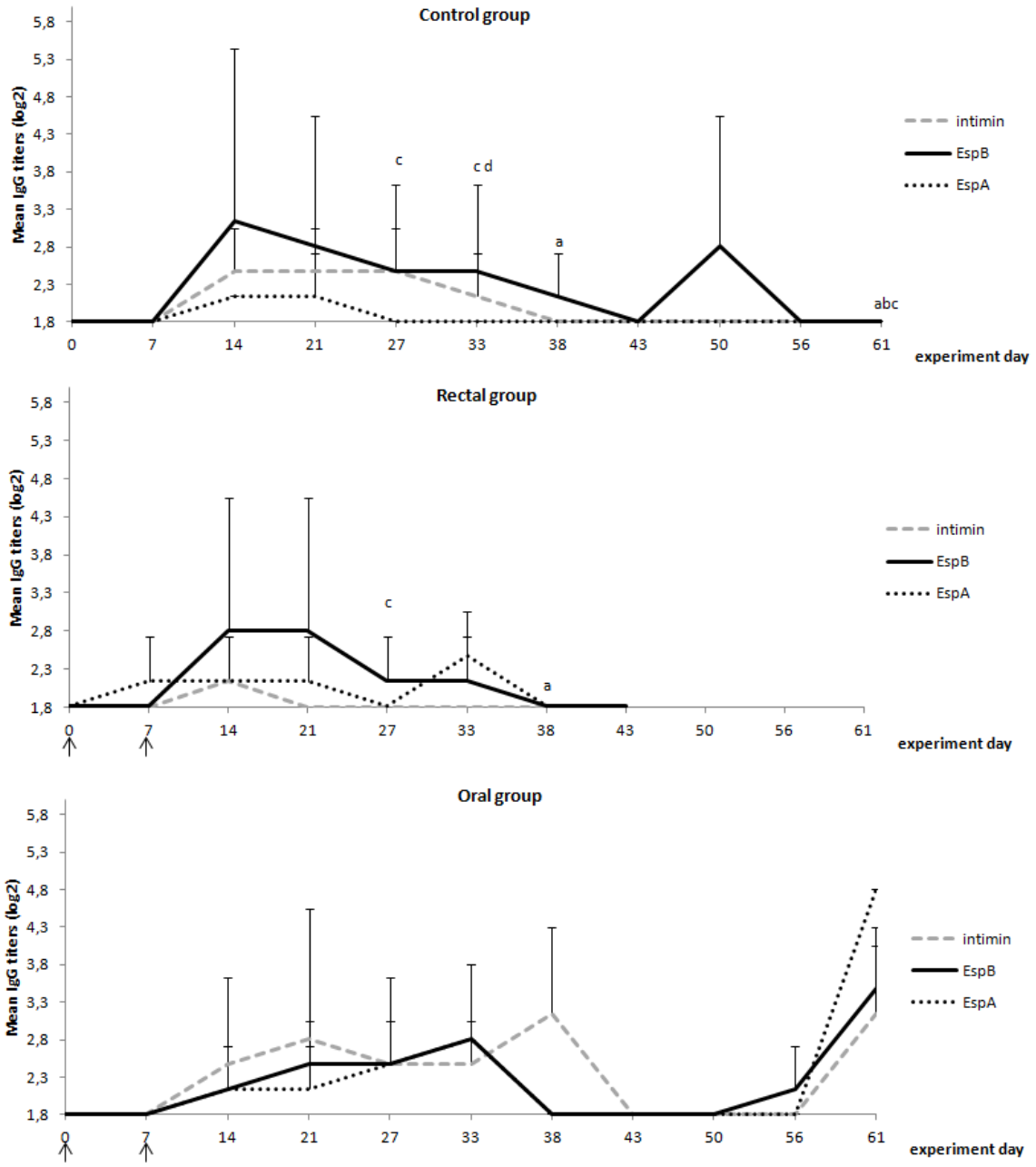

Figure 4.1: Mean IgG titers (log2) for the 3 treatment groups. Letters indicate a significant difference between groups for a designated time point; (a) significant differences in intimin responses compared to the oral group, (b) significant differences in EspB responses to the oral group; (c) significant differences in EspA responses to the oral group (c) and to the rectal group (d). Arrows indicate the time points of oral inoculation with bacteria.

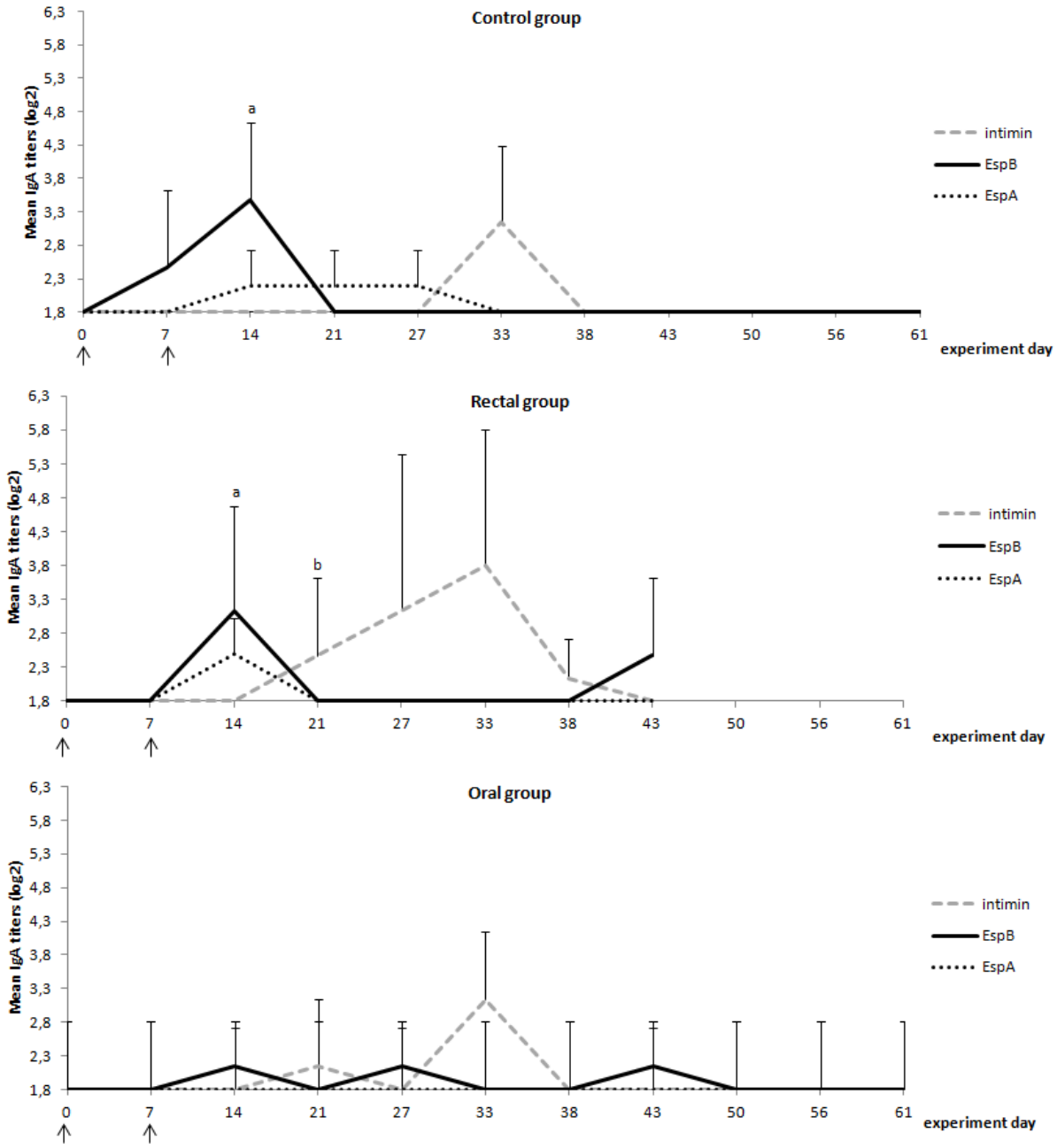

Figure 4.2: Mean IgA titers (log2) for the 3 treatment groups. Letters indicate a significant difference between groups for a designated time point; (a) significant differences in EspB responses compared to the oral group, (b) significant differences in EspA responses to the oral group. Arrows indicate the time points of oral inoculation with bacteria.

4.4.2. Excretion of E. coli O157:H7

For all 3 animals, fecal shedding heavily fluctuated between 10^{1} and $10^{5.5}$ CFU *E. coli* O157:H7/g feces when positive from day 1 until euthanasia at day 61 (Figure 4.3). The feces of animals 1, 2, and 3 were *E. coli* O157:H7 negative on 4 (25%), 1 (6.2%), and

5 (31%) of 16 sampling time points, respectively. Only 1 of 3 (33%) animals (animal 3) ceased shedding at day 50 and remained negative the end of the experiment (day 61).

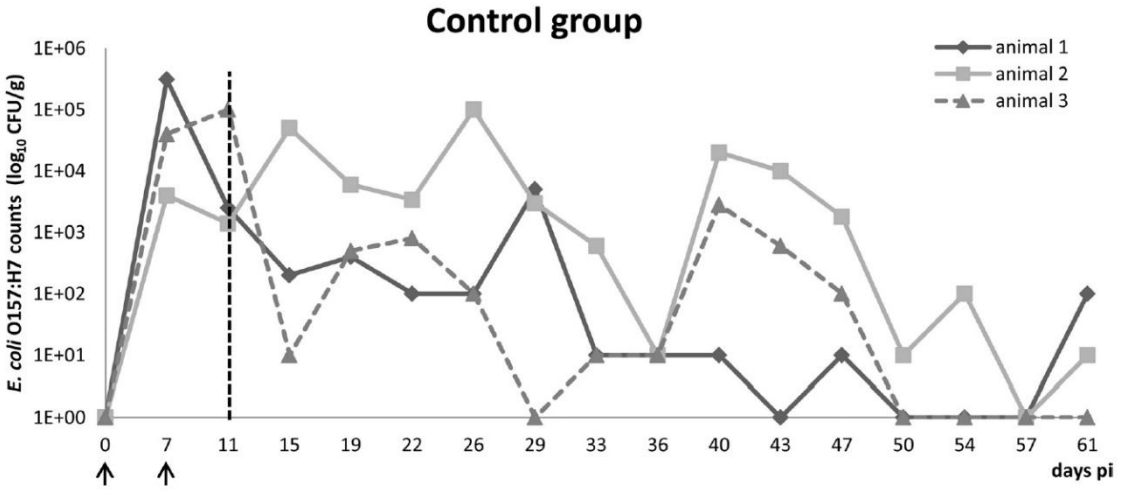

Figure 4.3: Excretion of *E. coli* O157:H7 in calves. Results are represented as real values of CFU/g feces for individual animals in the control group on a logarithmic scale. A value of 1E+00 corresponds to negative sampling after enrichment and IMS. The vertical dashed line at day 11 indicates the start of the treatment. The arrows indicate the starts of the first and second infections.

Also in the oral group, fecal *E. coli* excretion fluctuated heavily between 10^1 and 10^4, 10^1 and $10^{3.5}$, and 10^1 and 10^5 CFU *E. coli* O157:H7/g feces, for animal 1, 2 and 3, respectively, when positive (Figure 4.4). Also here one animal (animal 1) ceased shedding, but already from day 33 onwards. For animal 2, feces were negative on 4 of 16 (25%) sampling time points. On the other hand, the feces of animal 3 remained *E. coli* O157:H7 positive from day 1 until day 61.

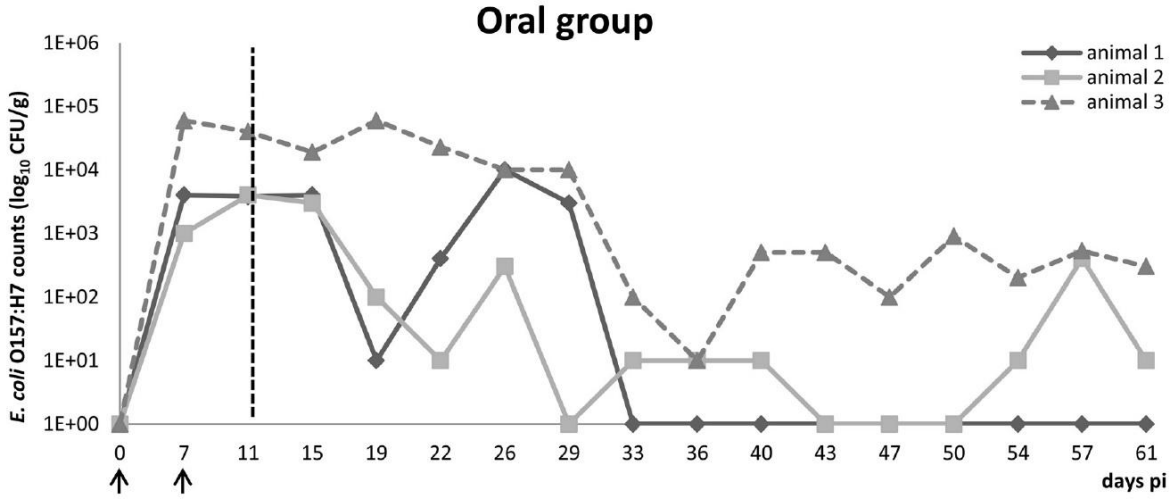

Figure 4.4: Excretion of *E. coli* O157:H7 in calves. Results are represented as real values of CFU/g feces for individual animals in the oral group on a logarithmic scale. A value of 1E+00 corresponds to negative sampling after enrichment and IMS. The vertical dashed line at day 11 indicates the start of the treatment. The arrows indicate the starts of the first and second infections.

Rectal administration of bLF gave a completely different result (Figure 4.). As in the oral and control groups, at day 1 large amounts of *E. coli* O157:H7 were found in all 3 animals in the rectal group ($10^{4.5}$,10^5, and 10^4 CFU, respectively). Hereafter, *E. coli* O157:H7 shedding constantly declined afterwards, resulting in 2 of 3 (66%) negative animals (animal 1 and 3) by day 19. The third animal (animal 2) became negative by day 33. Importantly, once negative, *E. coli* O157:H7 could not be isolated again in the feces of these animals.

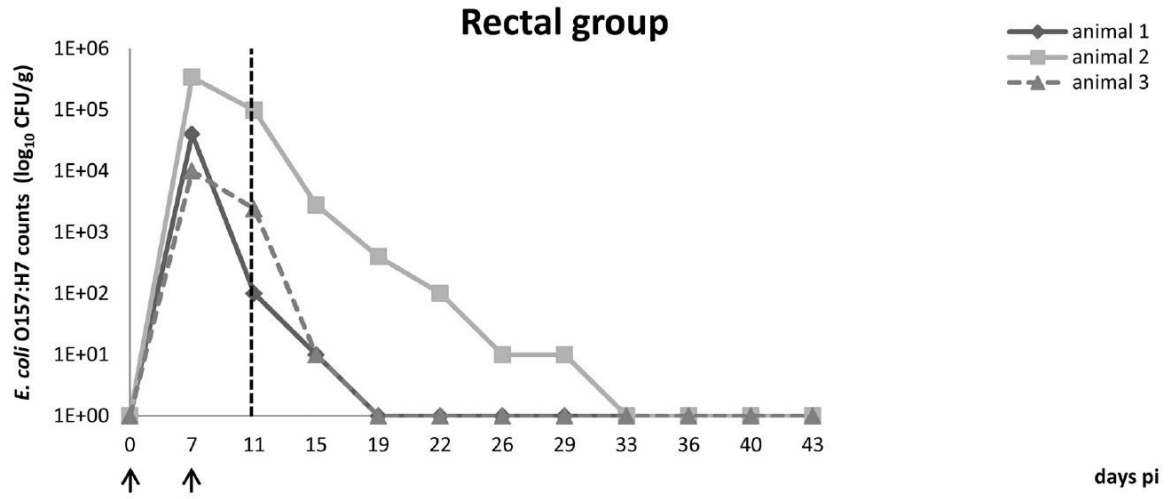

Figure 4.5: Excretion of *E. coli* O157:H7 in calves. Results are represented as real values of CFU/g feces for individual animals in the rectal group on a logarithmic scale. A value of 1E+00 corresponds to negative sampling after enrichment and IMS. The vertical dashed line at day 11 indicates the start of the treatment. The arrows indicate the starts of the first and second infections.

The treatment groups did not have a significantly lower mean *E. coli* O157:H7 CFU/g feces (AUC) than the control group (Figure 4.6). However, the P-value for the comparison between the rectal and the control groups equaled 0.0414, slightly below the Bonferroni-adjusted significance level of 0.05.

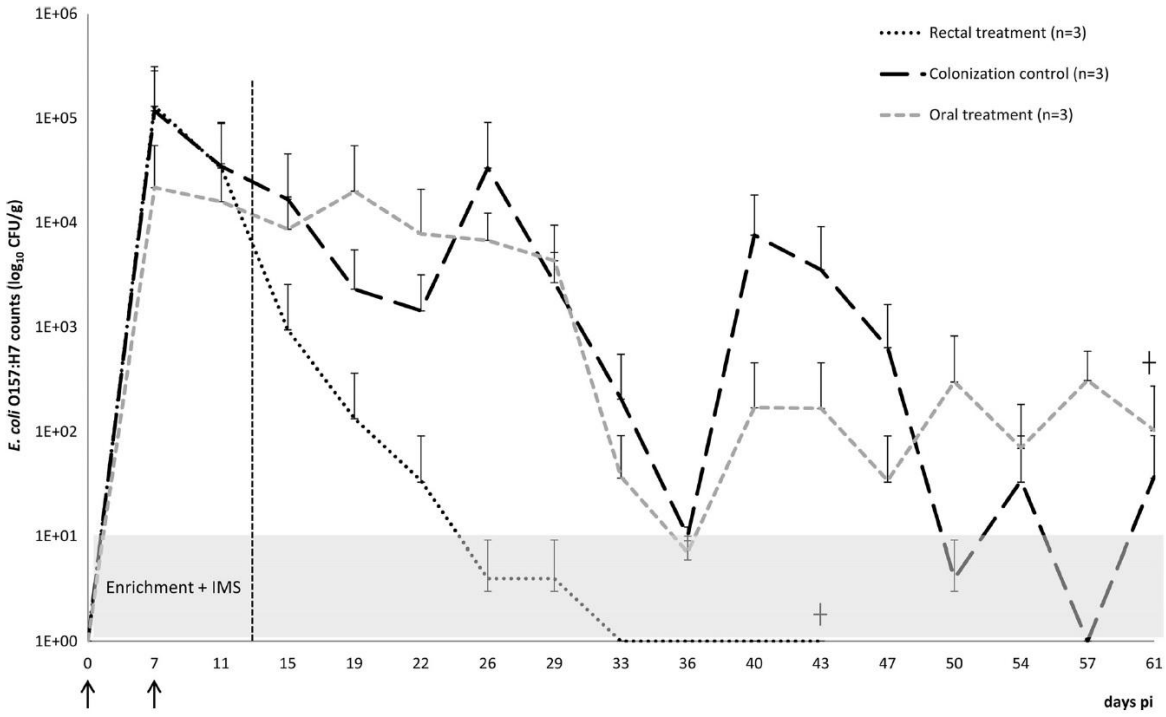

Figure 4.6: Results are presented as the mean real values of CFU/g feces ± SD on a logarithmic scale. A value of 1E+00 corresponds to negative sampling after enrichment and IMS. The vertical dashed line at day 11 indicates the start of the treatment. The arrows indicate the starts of the first and second infections.

4.4.3. Presence of E. coli O157:H7(Stx⁻) in intestinal tissues at euthanasia

E. coli O157:H7 (Stx⁻) could not be isolated from the intestinal tissues or contents of animals in the rectal group (Table 4.1). On the other hand, the bacteria could be isolated from the intestinal contents of 2 of 3 (66%) animals in the oral and control groups. In the control group, it was found in contents of the small intestine and rectum of 1 of 3 (33%) and 2 of 3 (66%) animals, respectively, with the largest amount in the ileum (6×10^2). Large intestinal contents were negative. For the oral group, *E. coli* O17:H7 (Stx⁻) was found in contents of the large intestine and rectum of 1 of 3 (33%) and 2 of 3 (66%) animals, respectively. The largest amount of *E. coli* O157:H7 (Stx⁻) was found in the rectum (3×10^2). Small intestinal contents were negative.

At euthanasia, *E. coli* O157:H7 (Stx^-) was never found in tissues of the jejunum and colon and was absent in all intestinal tissue suspensions of animals in the rectal group (Table 4.2). The bacteria were present in recto-anal tissue suspensions of 1 of 3 (33%) animals of the oral and of the control group and in tissue suspensions of ileum without PP of 2 of 3 (66%) animals in the control group.

Table 4.1. Number of E. coli O157:H7 CFU/g of intestinal content at euthanasia on day 43 (rectal group) or day 61 (oral and control groups)

		E. coli O157:H7 (Stx^-) CFU in intestinal contents of				
Group	Animal N°	jejunum	ileum	caecum	colon	rectum
Oral	1	<10	<10	<10	<10	1×10^1
	2	<10	<10	<10	<10	<10
	3	<10	<10	2×10^3	$1{,}1 \times 10^3$	3×10^2
Rectal	1	<10	<10	<10	<10	<10
	2	<10	<10	<10	<10	<10
	3	<10	<10	<10	<10	<10
Control	1	<10	<10	<10	<10	1×10^1
	2	<10	<10	<10	<10	<10
	3	1×10^2	6×10^2	<10	<10	1×10^2

Table 4.2. Number of *E. coli* O157:H7 (Stx$^-$) CFU/g of tissue at euthanasia on day 43 (rectal group) or day 61 (oral and control group)

		E. coli O157:H7 CFU in tissue suspensions of[a]	
Group	Animal N°	ileum-PP	Recto-anal junction
Oral	1	4×10^2	<10
	2	<10	<10
	3	3×10^2	1×10^2
Rectal	1	<10	<10
	2	<10	<10
	3	<10	<10
Control	1	<10	<10
	2	<10	1×10^2
	3	<10	<10

[a] The number of CFU in tissue was determined by direct plating. Jejunum with Peyer's patches (PP), jejunum without PP, ileum with PP, and colon were negative in all groups.

4.5 Discussion

Bullen et al. (1972) first demonstrated the *in vivo* effects of lactoferrin by using milk against enteropathogenic *E. coli* in the small intestines of guinea pigs. They found that the bacteriostatic effect of milk was mainly due to lactoferrin. Later, Wada et al. (1999) showed that the administration of bLF to *Helicobacter pylori*-infected mice resulted in a marked decrease of bacterial colonization and attachment to the gastric epithelium. More recently, we demonstrated clearance of *E. coli* O157:H7 (Stx$^-$) excretion from the intestine of sheep by oral administration of bLF (Yekta et al. 2011).The mechanisms behind this clearance are far from elucidated, but it might be expected that both immunomodulating and antibacterial effects contribute to the clearance. Since cattle are the main reservoir of *E. coli* O157:H7, we investigated in the present study the effects of rectal and oral administration of bLF on systemic antibody production and on fecal shedding and intestinal clearance of *E. coli* O157:H7 (Stx$^-$) in experimentally infected calves. As in our

previous sheep experiment, bLF was administered orally in a 10% bicarbonate buffer. This buffer closes the esophageal groove, allowing bLF to pass the rumen, reticulum, and omasum and be directly transported to the abomasum (Rosenberger et al. 1979). Delivering bLF directly to the abomasum could prevent it from being consumed by ruminal bacteria and enhance its degradation by pepsin in the abomasum. Pepsin can cut bLF into different peptides, of which one peptide, lactoferricin, is believed to be involved in many lactoferrin functions. It has been shown that lactoferricin can be a more potent antibacterial compound than the native protein (Gifford et al. 2005; Bellamy et al. 1992), but the immunomodulating effects of lactoferricin are not well described.

In the present study, we could not observe clear differences in production of systemic antibodies against *E. coli* O157:H7 proteins EspA, EspB or intimin for the different groups. The IgG responses against EspB were comparable between control, rectal and oral groups indicating bLF had not effect on EspB in this trial. IgG responses against EspA were present in both bLF groups but not in the control group, suggesting that bLF enhanced antibody responses against this molecule. Furthermore, bLF accelerated the appearance of an intimin-specific IgA response upon re-infection with one week. This indicates that bLF can, to a certain extent, modulate systemic immune responses. However, a distinct effect on these responses was not detected, suggesting that the real immunomodulating effect might be found in local responses. A study from Rybarczyk et al. (2015) revealed that rectal administration of bLF induced EspA- and EspB-specific IgA responses in rectal mucosal tissue. These mucosal antibodies were only detected in animals which received bLF treatment and can therefore be linked to the immunomodulating effect of bLF. Furthermore, these local antibodies may contribute to the clearance of *E. coli* O157:H7 (Stx^-) from the rectum, together with the antibacterial effect of bLF. Whereas in a previous study, oral bLF treatment of sheep reduced the amount and duration of *E. coli* O157 (Stx^-) fecal shedding, such an effect was not seen in the orally treated calves in this study. An explanation for this difference could be the different tissue tropisms of *E. coli* O157:H7 in sheep and calves. In calves, the terminal rectal mucosa, which is rich in lymphoid follicles, has been identified as the major site of *E. coli* O157:H7 colonization (Naylor et al. 2003; Naylor et al. 2005). In sheep, the bacteria seem to colonize the entire intestinal tract (Vande Walle, Yekta, et al. 2011; Yekta et al. 2011; La Ragione et al. 2009), even though slightly higher numbers could be seen in the ileum and large intestine (Aktan et al. 2007) or at the recto-anal junction

(Yekta 2011; Vande Walle et al. 2011). It is likely that the antimicrobial activity of orally administered bLF decreases from cranial to caudal in the gut so that an effect can be seen on *E. coli* O157:H7 colonization in the orally inoculated sheep, with a more equal colonization throughout the gut, compared to calves, in which the bacterium has a predilection for the recto-anal junction. This is supported by the following observation that only rectally administrated and not orally administrated bLF could clear the infection from calves, whereas orally administrated bLF could clear the infection in sheep. To our knowledge, a total clearance of EHEC in cattle using a rectal treatment had not yet been described. Naylor et al. (2007) and Rozema et al. (2009) reported different strategies for rectal treatment that could reduce but not clear the bacterial colonization.

Although antibody responses were detected in the three groups of animals, we do not believe that they play a major role in the clearance of bacteria, since a study by Joris et al. (2012) showed that responses against EspA, intimin, and Tir did not always occur during infection with EHEC. When responses against EspB occurred, the feces were positive for EHEC and the response lasted for several months. Therefore, responses against EspB might be used in the future as an indication of the EHEC-positive status of animals. Nevertheless, interpretation of these serum responses should be performed carefully, as we saw in this experiment that responses against EspB can disappear although animals are still shedding. Furthermore, we could not observe a clear effect of bLF on the antibody responses against EspA, EspB, and intimin. In a study by Yekta et al. (2011) with oral bLF treatment in sheep, there was no observed effect of bLF on the intimin responses. The EspA and EspB responses were significantly augmented by the high bLF dose. This result was not seen in our experiment with calves, although the same dose of bLF was used in our oral treatment group.

Although we were able to use only a limited number of laboratory animals, the results of this preliminary study are promising since we observed a complete clearance of EHEC after rectal administration of lactoferrin. Future investigations should address the limitations of this study related to sample size and could therefore be performed on the farm level.

4.6 Conclusions

To our knowledge, this is the first study demonstrating a reduction of *E. coli* O157:H7 (Stx^-) shedding by rectal administration of bLF. Moreover, the administration of this antimicrobial protein resulted in the clearance of rectal colonization. However, when bLF was administered orally, no clearance could be observed. This could indicate that orally administered bLF might not reach the recto-anal junction in a sufficient way. Although the results on bacterial shedding were encouraging, this could not be linked to the influence of bLF on the systemic immune response. This suggests that bLF could have local effects on the immune system which affect the clearance of the bacteria, which could explain the increase in intimin-specific IgA, observed following rectal treatment with bLF. Results also demonstrate that bLF might be an interesting tool to reduce the number of *E. coli* O157:H7-excreting cattle, thereby reducing the exposure of humans to this zoonotic pathogen. However, field experiments for further validation of this new strategy have to be performed.

4.7 Acknowledgments

The authors wish to thank Dr. M. J. Woodward for providing *E. coli* strain NCTC12900. This research was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (RF10/6233).

Chapter V: Experimental work

Effect of Stx2 and bLF on release of proinflammatory cytokines by rectal epithelial cells and intestinal explants from bovine origin inoculated with *Escherichia coli* O157:H7

Adapted from: Kieckens E., Rybarczyk J., Vanrompay D., Cox E. (2016). Effect of Stx2 and bLF on release of proinflammatory cytokines by rectal epithelial cells and intestinal explants from bovine origin inoculated with *Escherichia coli* O157:H7. (Manuscript submitted).

5.1 Abstract

Escherichia coli O157:H7 is prevalent in cattle worldwide, but mechanisms leading to persistent colonization, are still far from elucidated. Results in earlier studies indicate a downregulation of immunity by Type III secretion system (T3SS) effector proteins and Shiga toxins (Stx) (Chapter I and Chapter III). In previous studies, we demonstrated that bovine lactoferrin (bLF) can clear an *E. coli* O157:H7 infection in experimentally infected cattle (Kieckens et al. 2015), and that bLF seems to enhance a rectal mucosal IgA response (Rybarczyk et al. 2015). In this study, we analysed the release of proinflammatory cytokines upon *E. coli* O157:H7 infection by (i) bovine rectal epithelial cells and upon infection by (ii) explants of the recto-anal junction (RAJ), the most important colonization site in cattle, and by (iii) explants of ileal Peyer's patches (I+PP), shown to play a role in suppression of the immune response upon colonization. Furthermore, we analysed the effect of Shiga toxin 2 (Stx2) and bLF on this proinflammatory cytokine response. Our data in rectal epithelial cells show that incubation with *E. coli* O157:H7 strains reduces both IL-6 and IL-8 levels and increases TNF-α levels. These effects were even more pronounced when cells were pretreated with Stx2. bLF was able to restore the IL-6 but not the IL-8 levels and induced the release of supplementary TNF-α. Inoculation of tissue explants with *E. coli* O157:H7 (Stx^-) induced the secretion of IL-6 and IL-8 in the RAJ and of IL-8 in the I+PP, while reducing only IL-6 in the I+PP. No significant effects of pretreatment with Stx2 could be observed. bLF could again restore IL-6, but overstimulated IL-8 secretion. TNF-α was slightly decreased by *E. coli* O157:H7 (Stx^-) in RAJ and increased in I+PP explants.

5.2 Introduction

The prevalence of *E. coli* O157:H7 in cattle varies widely, depending on countries, regions, farms types (dairy versus beef), and herds. But a recent study in Belgium has reported an overall prevalence of 37.8 % cattle farms which are infected, with the highest prevalence for dairy cattle farms (61.2%, 30 of 49 farms) (Cobbaut et al. 2009). This shows that there is a significant risk for transmission of these bacteria to humans, where it can result in severe clinical signs and mortality. Many researchers have studied factors

that can influence colonization in cattle, but there are still a number of gaps that need to be elucidated to fully understand the mechanisms behind this colonization. Until now, there is no explanation why some animals within a herd shed high numbers of *E. coli* O157:H7 over longer time periods whereas others barely get infected and/or shed only small amounts of the bacteria. This effect might be linked to differences in immune responses and levels of cytokine production between animals.

Several studies have demonstrated that *E. coli* O157:H7 can modulate immune responses in a variety of ways. Recognition of H7 flagellin by TLR5 and of lipopolysaccharide (LPS) by TLR4 initiates proinflammatory responses, which can be downregulated by injection of Locus of Enterocyte Effacement (LEE) and non-LEE encoded proteins via the bacterial type III secretion system (T3SS). Indeed, Tir, EspB, NleB, NleC, NleD, NleE, NleH1 and NleH2, can all intervene in the NF-κB pathway decreasing the expression of pro-inflammatory cytokines IL-6, IL-8 and TNF-α (Hauf and Chakraborty, 2003; Wan et al. 2011; Vande Walle et al. 2013; Chapter 1). However, NleF can enhance the proinflammatory responses. Besides modulation via T3SS effector proteins, modulation of the immune system can also occur by Shiga toxins (Stx) 1 and/or 2 produced by enterohemorrhagic *E. coli* (EHEC) (Chapter I, section 2.8.1). Stx is capable of inducing IL-6, IL-8, and TNF-α production in bovine and murine macrophages and human intestinal epithelial cells (Menge et al. 2015; Tesh et al. 1994; Smith et al. 2003). The Stx inhibit protein synthesis by acting on the 28S rRNA of the 60S ribosome of eukaryotic cells (Endo et al. 1988). This results in cellular apoptosis, which initiates the inflammatory response and influx of leukocytes (Smith et al. 2003). Stx2-producing EHEC strains are more frequently linked to severe disease in humans than strains producing Stx1 or a combination of both (Fuller et al. 2011). Moreover, $Stx2^+$ EHEC are shown to suppress cellular immune responses, which might facilitate long-term colonization and re-infection (Hoffman et al. 2006).

In cattle, the recto-anal junction (RAJ) is the predominant colonization site of *E. coli* O157:H7 (Naylor et al. 2003). We hypothesized that the epithelium of the RAJ could play a major role in the suppression of the immune system by *E. coli* O157:H7, but an RNA-Seq experiment performed on mRNA extracted from the RAJ and ileal Peyer's patches (I+PP) of animals that were infected and re-infected with a Shiga toxin-negative (Stx^-)-strain could not confirm this hypothesis (Chapter III). On the contrary, we found a

weighty effect on the gene expression in the I+PP, while the RAJ was almost unaffected. A limitation of our study using the RNA-Seq technique is that changes in mRNA expression were only determined at a specific time point (14 days post infection or re-infection). Most likely activation or suppression of proinflammatory responses by EHEC changes in time, explaining why we could not observe effects on mRNA of these cytokines, which are short-lived, using the RNA-Seq method. In the present study tissue explants or cell cultures were used and incubated with *E. coli* O157:H7 in the presence of absence of Stx and/or bovine lactoferrin (bLF). Incubation of explants or cells with bacteria was performed during six hours, a period long enough to measure proinflammatory cytokine responses on protein level. We analysed the cytokine response of RAJ explants, but because of the extensive effect on mRNA expression in I+PP tissues, also of I+PP explants. As the predilection of EHEC in cattle for the recto-anal epithelium was demonstrated (Naylor et al. 2003), we also established bovine primary rectal epithelial cell cultures, which were used to analyze their role in proinflammatory responses of *E. coli* O157:H7 as well as to determine the effect of Stx2 on their interaction with *E. coli* O157:H7. Since there are indications that Stx2 might facilitate colonization of *E. coli* O157:H7, Stx2 pretreatment of rectal epithelial cells was included.

Previous studies in our group showed that bLF was capable of clearing *E. coli* O157:H7 from the rectal mucosa when administered topically (Chapter IV). This was accompanied with the induction of mucosal IgA responses (Rybarczyk et al. 2015). Since IL-6 is known to enhance IgA secretion, we analyzed the effect of bLF on the proinflammatory cytokine responses of tissues explants and cell cultures (Mowat 2003).

5.2 Materials and methods

5.2.1 Cell culture of bovine rectal epithelial cells

Rectal specimens were obtained from freshly slaughtered cattle (18-24 months old) of different breeds from a local slaughterhouse (Flanders Meat Group, Zele, Belgium). The rectal tissues were rinsed with cold phosphate buffered saline (PBS, pH 7.2, 4°C) supplemented with 100 U/ml penicillin (Sigma-Aldrich, Diegem, Belgium), 100 µg/ml streptomycin (Sigma-Aldrich) and 25 µg/ml gentamicin (Thermo Fisher Scientific). Further, the rectal specimens were placed in ice-cold Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific) supplemented with 100 U/ml penicillin (Sigma-

Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 2.5 µg/ml amphotericin B (Sigma-Aldrich) and 25 µg/ml gentamicin (Thermo Fisher Scientific) and transported on ice to the laboratory.

Rectal crypts were isolated from rectal specimens as described (Bridger et al. 2010; Kaushik et al. 2008; Sheng et al. 2011), with minor modifications. Briefly, mucosal tissue was separated from the lamina propria by scraping with a sterile glass slide, homogenized by a razor blade and centrifuged (5 min, 130 x g, 4°C) in HBSS (4°C) three times to remove the upper mucus containing layer. The remaining pellet was used for enzymatic digestion (60 min, 100 rpm, 37°C) carried out in a solution containing Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific), 1% (v/v) heat-inactivated fetal calf serum (FCS), 100U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 2.5 µg/ml amphotericin B (Sigma-Aldrich), 25 µg/ml gentamicin (Thermo Fisher Scientific) and 100 U/ml collagenase (Sigma-Aldrich). To disintegrate crypt clots, the suspension was passed through a 0.9 x 50 mm needle (Beckton Dickinson, Erembodegem, Belgium), whereafter it was centrifuged 5 times (5 min, 50 x g, 4°C) with DMEM containing 2% sorbitol (w/v) to enrich the crypts and separate them from the most bacteria and single cells including fibroblasts. Rectal crypts formed a pellet while the supernatant containing single cells and debris was discarded after each centrifugation step. At the end the crypts were washed with HBSS (3 min, 65 x g , 4°C) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 25 µg/ml gentamicin.

Isolated crypts were seeded at a density of 200-250 crypts per well into 24-well culture plates pre-coated with rat tail collagen (5 µg/cm^2; Sigma-Aldrich) and cultivated in 37°C, and 5% CO_2, in complete culture medium containing DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 10 ng/ml epidermal growth factor (Sigma-Aldrich), and 10 µg/ ml bovine insulin (Sigma-Aldrich). For the first 24 h, 10% FCS was added to enhance the attachment of the crypts. After that only 1% of FCS was used to reduce the amount of fibroblasts. The growth of fibroblasts was additionally inhibited by light trypsinization (0.025% Trypsin-EDTA for 2-3 min at 37°C, every 2 days). The epithelial nature of the culture was confirmed by detecting cytokeratin by immunofluorescence staining with the anti-pan cytokeratin antibody, clone C11 (C2931; Sigma-Aldrich) as described by Hoey

et al. 2003 and Kaushik et al. 2008. A monoclonal anti-vimentin antibody (clone V9) (V6389; Sigma-Aldrich) was used to detect fibroblasts in the cell culture.

5.2.2 Generation of bovine intestinal tissue explants

Three Holstein-Friesian calves (±12 months old), seronegative for EspA, EspB and intimin, were euthanized and tissues from the ileal Peyer's patches and the RAJ were isolated. The tissues were cut in pieces of 1 cm² and washed using ice-cold sterile PBS (2°C, pH 7.4). Subsequently, the pieces were immersed with cold Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher Scientific) during 30 minutes. Then, the tissues were placed with the mucosal side up on biopsy foam pads (Leica Biosystems, Nussloch GmbH, Germany) in 6-well tissue culture plates (Sigma-Aldrich) with 5 ml RPMI 1640 medium per well and incubated at 37°C, 5% CO_2.

5.2.3 Inocula: E. coli O157:H7 strains, bovine lactoferrin and purified Stx2

Two bovine EHEC field isolates, strain 147 (O157:H7, $Stx2^+$) and strain 220 (O157:H7, $Stx1^+/Stx2^+$), and one *E. coli* isolate of human origin (strain NCTC12900; O157:H7, Stx^-; Dibb-Fuller et al. 2001) were used. The bacteria were grown overnight in Luria-Bertani (LB) broth with aeration (200 rpm) at 37°C, harvested by centrifugation (11,337 x g, 5 min, 4°C), and re-suspended in DMEM.

Bovine lactoferrin (bLF) with 92% purity and 16% iron saturation (Ingredia Nutritional, Arras, France) derived from bovine milk, was used. Purified Stx2 was obtained from Toxin Technology Inc (Sarasota, USA) with a purity of 50 %.

5.2.4 Incubation of primary rectal epithelial cell cultures or tissue explants with Stx2, EHEC and/or bLF

Rectal epithelial cells were pre-treated with 10 ng/ml of purified Stx2 or 0.05 mg/ml of bLF, during 24 hours. The next day, all medium was replaced by the corresponding medium with or without one of the three bacterial strains (10^6 CFU/ml medium), eventually supplemented with Stx2 (only for the Stx^- strain) and bLF (triplicates of each condition). After six hours of incubation (37°C; 5% CO_2) the supernatant was collected and stored at -20°C for further analysis.

Six explants of the RAJ and Ileal Peyer's patches in RPMI 1640 medium were cultured during six hours with 10^6 CFU/well of strain NCTC12900 (Stx^-), 10^6 CFU/well

of strain NCTC12900 (Stx^-) + 0.05 mg/ml bLF, 10^6 CFU/well of strain NCTC12900 (Stx^-) + 5 ng/ml Stx2, 0.05 mg/ml bLF, or 5 ng/ml Stx2 only. Explants of both tissues incubated in RPMI 1640 medium served as controls. After six hours of incubation (37°C; 5% CO_2) the supernatant was collected and stored at -20°C until further analysis with cytokine-specific ELISA's.

5.2.5 Cytokine analysis

The IL-6, IL-8 and TNF-α concentrations in supernatants were determined by ELISA using either the Bovine IL-6 sandwich ELISA Reagent Kit (detection range 80-5000 pg/ml; ESS0029; Thermo Scientific), the Bovine IL-8 competitive ELISA kit (detection range 50-2000 pg/ml; MBS701879; MyBiosource, San Diego, USA) or the Bovine TNF-α sandwich ELISA kit was obtained from RayBio (detection range 125-8000 pg/ml ELB-TNFa; RayBiotech Inc, Norcross, USA) without modifications to the manufacturer's protocol. The cytokines were chosen because TNF-α and IL-6 together with IL-1 are essential in the acute phase response, initiating the innate immune response, while chemokine IL-8 causes influx of immune cells at the site of infection, which is important for a subsequent adaptive immune response. Standards and supernatant of samples from three animals were tested as technical duplicates. A standard curve was generated by plotting the average absorbance obtained for each standard concentration on the Y-axis vs. the corresponding cytokine concentration on the X-axis.

5.2.5 Statistical analysis

GraphPad Prism was used for statistical analysis and graphic design of figures. Data from cytokine ELISA's for rectal epithelial cells were analyzed using Tukey's multiple comparisons test to identify statistic significant differences ($p<0.05$) between conditions for each *E. coli* strain. The data for tissue explants were analyzed using Tukey's multiple comparisons comparing the different conditions per tissue type.

5.3 Results

5.3.1 Cytokine release by rectal epithelial cells

Following optimization of the primary bovine rectal epithelial cells cultures (Figure 5.1), the cultures were used as an *in vitro* model for studying the interaction of Stx2, bLF and *E. coli* O157:H7 strains with bovine primary epithelial cells.

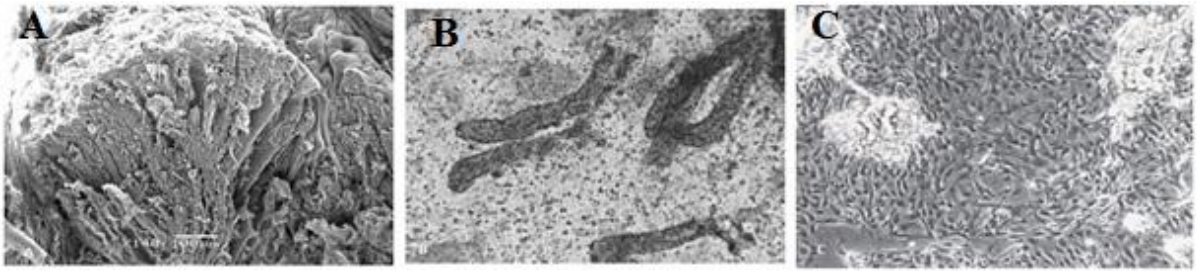

Figure 5.1: Microscopic pictures of the RAJ. From left to right: (A) Rectal crypts (SEM); (B) Enzymatic digestion of rectal crypts (LM); (C) Monolayer of rectal epithelial cells after digestion with collagenase (LM).

We determined the cytokine concentrations in the supernatants using IL-6, IL-8 and TNF-α specific ELISAs (Figure 5.2).

Incubation with *E. coli* O157:H7 strains reduced the IL-6 secretion in the supernatants compared to control cells (which were set at 100%), while pretreatment and supplementation with bLF increased IL-6 levels above the control cells. Only pretreating the cells with bLF partially restored the IL-6 concentration. Pretreating the cells with Stx2 on the contrary decreased the IL-6 secretion when subsequently inoculated with bacterial strains, which also produced Stx. Supplementing the Stx^- strain with Stx2 after a pretreatment with Stx2 diminished the IL-6 levels below the detection limit.

Only the *E. coli* O157:H7 $Stx1^+Stx2^+$-strain clearly reduced IL-8 levels and pretreatment with Stx2 could not aggravate this reduction. An increase in IL-8 was observed when the $Stx2^+$-strain was pretreated with Stx2. Furthermore, bLF was unable to restore IL-8 concentrations and even reduced it to the lowest levels. The IL-8 levels for the Stx^- strain were comparable in all Stx2 and bLF groups.

TNF-α levels were increased by the Stx^- strain and after pretreatment with Stx2 and bLF for all strains. Inoculation with Stx^+ strains decreased the TNF-α level to below

detection levels as treatment with bLF did. The effects of the Stx^- strain with and without pretreatment with Stx2 on TNF-α were comparable to the Stx^+ strains.

In conclusion, incubation with Stx^+ strains could reduce IL-6 and TNF-α concentrations, whereas for IL-8 this was only the case for the double Stx^+ strain. Pretreatment with Stx2 increased the effect on IL-6 but reduced the effects on IL-8 and even reverted the effect on TNF-α. Effects of bLF seem to be independent of the strains used. Pretreatment of cells with bLF reduced the decrease in IL-6, but not in IL-8, whereas it enhanced TNF-α production induced by the Stx^+ strains. However, adding bLF also to the bacterial strains, restored IL-6 completely, had no additional effect on IL-8 and reduced TNF-α below the detection limit. The effect of bLF on IL-6 might be consistent with the local mucosal IgA response following rectal bLF treatment of calves rectally colonized with O157:H7 *E. coli* (Rybarczyk et al. 2015).

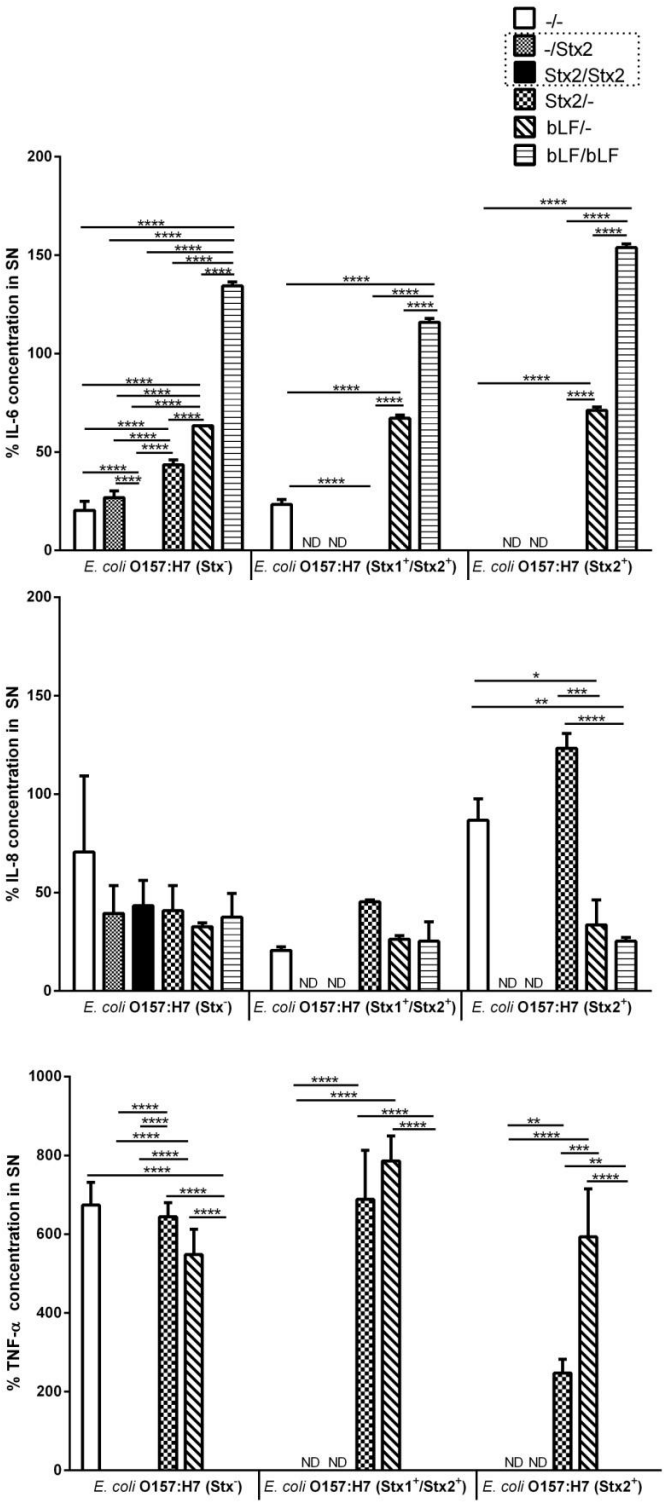

Figure 5.2: Cytokines secreted in supernatant of rectal epithelial cells after incubation with Stx2 or bLF. Stx2 inhibited the release of IL-6 and IL-8, while bLF partly restored the release of IL-6 and TNF-α. Data are presented as % values relative to the cytokine concentration in untreated cultures (set at 100%). Conditions decribed before / indicate pretreatment and after / treatment together with inoculation of the strain. Missing values are caused by concentrations below the detection limit of the ELISA. Error bars indicate standard deviations. ND indicates conditions that were not determined (only tested for the *E. coli* O157:H7 (Stx$^-$) strain). Significant differences were indicated with *(*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$)

5.3.2 Cytokine release by bovine intestinal explants

Although EHEC directly interact with mucosal epithelial cells, immune cells in close proximity to the epithelum might also influence the innate immune response. Therefore we assessed the effect of a Stx$^-$ *E. coli* strain, Stx2 and bLF on tissue culture explants. Six bovine intestinal explants of I+PP and the RAJ were cultured during six hours in the presence of *E. coli* O157:H7 (Stx$^-$), *E. coli* O157:H7 (Stx$^-$) + Stx2, *E. coli* O157:H7 (Stx$^-$) + bLF, bLF, and Stx2. The IL-6, IL-8 and TNF-α production was determined by cytokine-specific ELISA's (Figure 5.3).

In contrast to the rectal epithelial cell culture, where all three cytokines were reduced by adding Stx2 to the Stx$^-$ *E. coli* strain, the cytokine levels did not change in culture supernatant of RAJ. Furthermore, adding bLF to the Stx$^-$ *E. coli* strain only had a minor effect. Whereas the bacteria non-significantly increased IL-6 and non-significantly reduced TNF-α, bLF seemed to normalize the concentration of both cytokines to basal levels (= 100%; control tissues incubated in cell medium). However, for IL-8 the opposite was observed. The infection slightly increased the IL-8 levels, but bLF increased IL-8 levels almost two-fold. The effects of bLF treatment are opposite to what was observed for rectal epithelial cells, suggesting an effect of immune cells in the complex tissue environment of explants.

For the I+PP explants, no effects were seen on levels of IL-8 and TNF-α, whereas IL-6 was reduced by infection with the Stx$^-$ *E. coli* strain in the presence or absence of Stx2. Adding bLF to the Stx$^-$ *E. coli* strain slightly increased the concentration.

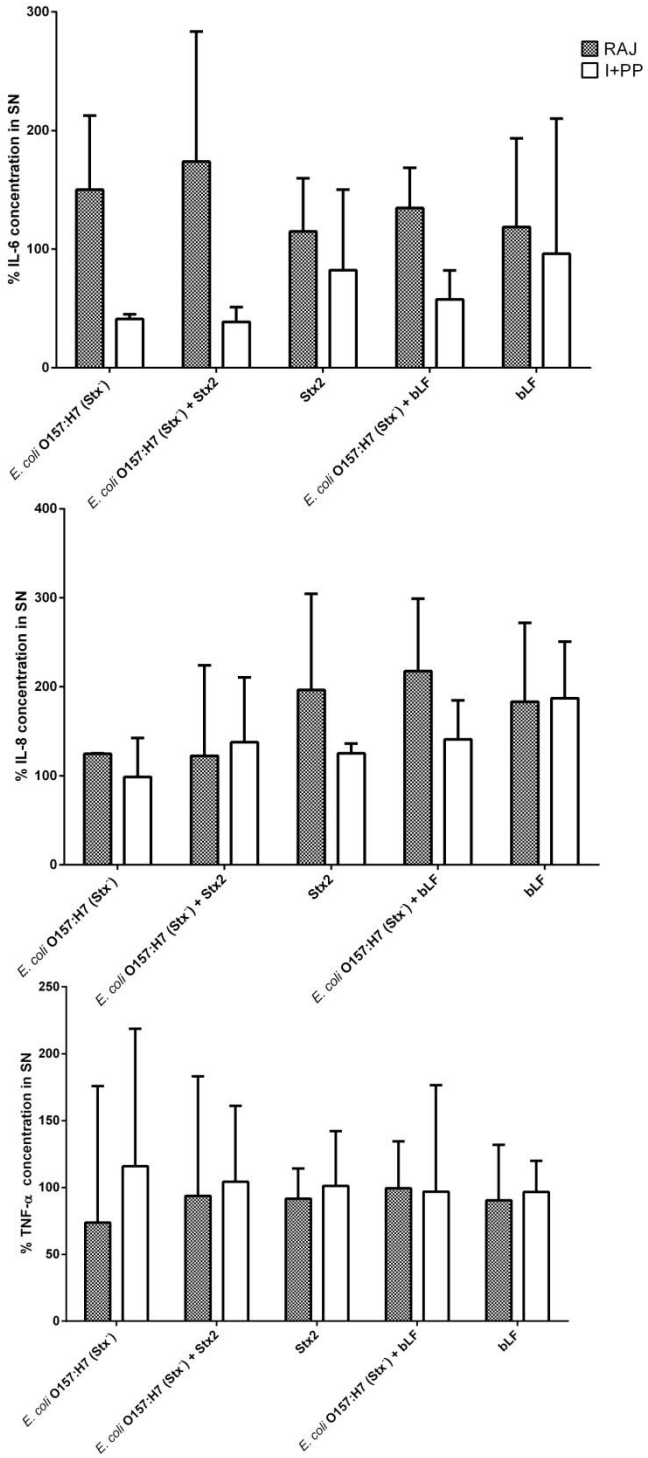

Figure 5.3: Release of IL-6, IL-8 and TNF-α from RAJ (n=6) and ileal Peyer's patches (I+PP) explants (n=6). The IL-6 and IL-8 concentration in RAJ and IL-8 concentration in I+PP was increased, but IL-6 decreased in the I+PP as a consequence of the *E. coli* O157:H7 (Stx$^-$) infection. bLF restored IL-6, but overstimulated IL-8 secretion. The TNF-α level was lowered in the RAJ but slightly increased in the I+PP upon *E. coli* O157:H7 (Stx$^-$) infection. Data are presented as % values relative to cytokine concentrations in supernatant of untreated explants (set at 100%). Error bars indicate standard deviations. Significant differences between conditions per tissue type were not found.

5.4 Discussion

Our data demonstrate that the immune system of calves infected with *E. coli* O157:H7 can be suppressed after a first infection (Chapter III) and that bLF could have a positive effect by enhancing the protective immunity of animals against *E. coli* O157:H7 colonization (Rybarczyk 2016). To gain more insight in the possible mechanisms of bLF and the effect on the epithelium and more complex tissues, we performed *in vitro* experiments using primary rectal epithelial cells and tissue explants.

Human colon epithelial cells are to be programmed to provide a set of signals for the activation of the mucosal inflammatory response in the earliest phases after microbial invasion, by upregulation of IL-8 and TNF-α as a response to infection with enteroinvasive *E. coli* (Jung et al., 1995). This raised the question if this could be confirmed for bovine rectal epithelial cells and EHEC. Therefore, we investigated the cytokine release of bovine primary rectal epithelial cells. We focused on the release of IL-6, IL-8 and TNF-α because these cytokines are known to play key roles in the early phase of the immune defence against bacterial pathogens. Hereafter, we investigated the cytokine release of tissue explants of the RAJ and I+PP to complement our data from the RNA-Seq experiment. These tissue explants form a complex environment in which mucosal epithelial cells interact with lymphocytes and antigen presenting cells.

IL-6 is produced by various cell types as T-cells, B-cells, dendritic cells, macrophages and monocytes (Mihara et al. 2012) and is one of the most important cytokines produced in the intestinal mucosa during inflammation because of its proinflammatory and anti-inflammatory effects (Pritts et al. 2002) by decreasing the effect of TNF-α and inducing epithelium repair (Kuhn et al. 2014; Scheller et al. 2014). IL-6, together with IL-1 are essential in the acute phase response, initiating the innate immune response (Striz et al. 2014). IL-6 is required for antibody production by B-cells and is involved in proliferation and differentiation of helper T-cells. IL-6 also promotes IL-4 induced T_H2 differentiation and inhibits IL-12 induced T_H1 differentiation and is involved in T_H17 induction (Mihara et al. 2012). It is important for inducing production of IgA by B-cells (Macpherson et al. 2008), and is known to enhance IgA secretion (Mowat 2003). bLF might modulate the host immunity by affecting this IL-6 release. Only in RAJ tissue explants, we detected an increase in IL-6 upon bacterial exposure, which indicates the protective effect of IL-6 against pathogenic threats. In contrast, rectal epithelial cells and

I+PP explants showed a reduction in IL-6 concentration. The opposite effect on IL-6 concentration between RAJ explants and rectal epithelium indicates that immune cells, which are only present in the complex explants, play a role. The effect in the I+PP is parallel to our data from Chapter III, showing a suppression of proinflammatory immune responses. The increase of IL-6 in RAJ explants effect was less pronounced when bLF was added to the bacteria, suggesting an attenuation of the immune response by bLF. This is congruent with a study in the human monocytic cell line THP-1 in which bLF suppresses the release of IL-6, as a reaction on the addition of bacterial LPS to the cell medium (Mattsby-Baltzer et al. 1996). This effect of bLF is due to a decreased LPS-induced binding of NF-κB to the TNF-α promoter, which reduces the IL-6 level (Haversen et al., 2002). Also in I+PP explants, bLF seemed to revert IL-6 secretion towards baseline levels suggesting a normalization of the immune response. A similar phenomenon was described in a study demonstrating the effect of bLF on immune cells. Treatment with bLF resulted in an expression of IL-6, IL-1, IL-18 and TNF-α that was restored to the baseline (Elass et al. 2002; Legrand & Mazurier 2010). We observed that if bLF was added to *E. coli* O157:H7 or used as a pretreatment, the effect of the bacteria was less pronounced. This could be due to the immunomodulating effect but also to the bactericidal effect of bLF.

IL-8 is a chemokine that causes influx of immune cells, mainly neutrophils but also T-cells, at the site of infection which is important for a subsequent adaptive immune response (Palomino & Marti 2015). EHEC can induce *in vitro* a potent proinflammatory response in the intestinal epithelium by secretion of IL-8 (Dahan et al. 2002). This secretion is due to the involvement of MAPK, AP-1, and NF-κB signalling pathways. Also EAEC can induce IL-8 secretion by intestinal epithelial cells in a rapid way by contact with flagellin (Steiner et al., 2000). bLF can counteracted this pathway as it inhibits the flagellin-induced activation of the NF-κB pathway (Inubushi et al. 2012). In the present study, RAJ tissues showed slightly increased IL-8 levels for all six *E. coli* O157:H7 (Stx^-) inoculated tissues, comparable to the results of Dahan et al. (2002) and Steiner et al. (2000). Also for ovine I+PP explants induction of IL-8 by *E. coli* O157:H7 infection was demonstrated (Atef Yekta 2011). Stx2 and bLF increased IL-8 levels even more. That Stx2 can induce IL-8 is not unexpected since studies using a human colonic epithelial cell line (Hct-8) showed that the active Stx A-subunit is capable of increasing

IL-8 (Thorpe et al. 1999). Less expected was the increase of IL-8 by bLF. This occurred when added to RAJ but also to I + PP.

The proinflammatory role of TNF-α is important in the host immune response to a number of microbial pathogens, including viruses, bacteria and parasites. It can be induced upon stimulation with bacterial LPS (Pawlaczyk et al. 2008). TNF-α is produced by activated monocytes, macrophages, lymphocytes and epithelial cells in response to an infection (Gardam et al. 2003; Jung et al. 1995). Aderka et al. (1989) has shown that IL-6 can inhibit the TNF-α production causing an anti-inflammatory effect on the immune system. The inhibitory action of IL-6 on TNF-α production is consistent with a predominantly anti-inflammatory role of IL-6 in the intact organism. In rectal epithelial cells, we observed an increase in TNF-α whereas a non-significant decrease was observed for the RAJ tissue. These opposite results again indicate a possible effect of immune cells which are only present in the RAJ tissue. This effect might be the result of partial inhibition of TNF-α secretion by a bacterial protein, as has been described for some enteropathogenic *E. coli* or *Yersinia enterocolitica* strains (Wilson et al. 1998).

A difficulty of this study is the fact that we could only investigate the cytokine levels after six hours of incubation with *E. coli* O157:H7, as longer incubation times are not possible due to bacterial overgrowth in cell and tissue cultures and degradation of explants. Analyzing later time points might affect the outcome since kinetics are important for cytokine effects. We investigated protein levels in supernatants which probably does not change so quickly as mRNA (Chapter III). Nevertheless, an added value for these data could be confirmation on mRNA level with PCR for different time points, taking into account the short stability of mRNA transcripts.

During the setup of this study, we were able to establish a good procedure for the isolation and maintenance of rectal epithelial cells, which was necessary since no permanent cell line of bovine rectal epithelial cells is available. This makes future studies about the interaction of cells with bLF more attractive.

5.5 Conclusions

Some of the data in this study are in line with suppression of the immune response by *E. coli* O157:H7 as IL-6 and IL-8 levels were found to be reduced in rectal epithelial cells and IL-6 levels in I+PP explants. bLF, a well studied immune-modulator, seems to restore the affected IL-6 cytokine levels to normal levels, promoting the natural homeostasis of the host. It remains unclear if this bLF effect is due to immunomodulation of the cells or bactericidal effects.

Chapter VI: Experimental work

Effect of bovine lactoferrin on release and bioactivity of Shiga toxins from different *Escherichia coli* O157:H7 strains

Adapted from: Kieckens E., Rybarczyk J., Barth S., Menge C., Cox E., Vanrompay D.(2016). Effect of lactoferrin on release and bioactivity of Shiga toxins from different *Escherichia coli* O157:H7 strains. Accepted in Veterinary Microbiology.

6.1 Abstract

Prevention of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infections and of their severe clinical sequelae in humans remain to be a current challenge. Administration of bovine lactoferrin (bLF) proved to be effective in clearing EHEC from the bovine intestine, an important EHEC reservoir, suggesting that bLF may also be beneficial in human application against EHEC infections. To estimate the biological safety of this approach, we analysed the effects of bLF on the main EHEC virulence factor, Shiga toxin (Stx). We quantified the release of Stx 1 and 2 from two O157:H7 EHEC strains ($Stx1^+Stx2^+$ and $Stx2^+$ producing, respectively) cultured in the presence of bLF using ELISA assays and assessed cytotoxic effects of bLF on Vero cells and on co-cultures of EHEC on Vero cells. Effects of bLF on the stability of Stx2 were investigated using Western blotting. ELISA results indicate a bLF concentration-dependent decrease of active, cell-free Stx2, but not Stx1 in EHEC cultures. High concentrations (100 and 50 mg/ml) of bLF resulted in significantly reduced ($p<0.05$) metabolic activity rates of Vero cells, whereas a concentration of 10 mg/ml bLF was considered non-toxic for Vero cells. At concentrations of 1 or 0.1 mg/ml, bLF mitigated the verocytotoxicity of EHEC strains in a co-culture model up to 48 hours after inoculation. For colonizing bacteria only, cytotoxicity could be significantly reduced by 10 and 1 mg/ml bLF during 48 hours. This effect of bLF at least partly results from degradation of the Stx2 receptor-binding B-subunit.

6.2 Introduction

Because of the potentially severe and long-lasting consequences for affected patients and the significant costs for health-care and economy, reduction of enterohemorrhagic *Escherichia coli* (EHEC) in prevalence and numbers at farm level and the mitigation if not prevention of EHEC-induced diseases in humans remain to be a current challenge for veterinary and human medicine (Elbasha et al. 2000). Every year, several small-scale outbreaks of EHEC O157:H7 are reported worldwide, repeatedly resulting in the need to hospitalized patients. In 2011, Germany and other European countries experienced an unprecedented outbreak caused by a highly virulent *E. coli* strain, which had acquired the capability of Shiga toxin (Stx) production by gene transfer. Subsequent withdrawal of food from the market and export bans had a major negative economic impact (Karch et

al. 2012). Clinical manifestations of human EHEC infections such as diarrhea, hemorrhagic colitis (HC) and, most importantly, the hemolytic uremic syndrome (HUS) leading to chronic renal failure or even death of the patient, pose a significant burden for the health care system (Sockett et al. 2014). Since the use of antibiotics is contra-indicated, because of the risk to aggravate the disease due to a higher release of Stx by the bacteria, treatment of infected patients is limited to supportive care. Consequently, there is an urgent need for curative therapies as well as effective prevention measures (Spacek et al. 2004).

Innovative strategies for effectively reducing EHEC carriage in ruminants would diminish the public health risk. Several studies were performed in cattle, the main reservoir of EHEC, in order to gain more insight into the prevalence and the colonization pattern of the bacteria. EHEC do not cause severe disease in cattle due to the distinct tissue distribution pattern of Stx receptors in the kidneys and the absence of receptors on blood vessels (Hoey et al. 2002). Shedding by cattle is intermittent, but the infection persists on farms in animals and their environment (Wells et al. 1991). A seasonal effect with consistently higher detection rates during spring and summer (Chapman et al. 1997) makes prevalence studies complex and hampers the development of reduction strategies. Different research groups have tried to reduce the environmental contamination by reduction of fecal shedding. Vaccination (Dziva et al. 2007; van Diemen et al. 2007; Van Donkersgoed et al. 2005), probiotic treatment (Brashears et al. 2003), bacteriophage administration (Sheng et al. 2006) and diet modification (Callaway et al. 2011) were of only limited success, however. A study using the host-specific iron-binding glycoprotein bovine lactoferrin (bLF) in ruminants was the only one showing a complete clearance of the bacteria from the host (Kieckens et al. 2015). The superior effect of bLF compared to other strategies might be due to the multiple antibacterial activities of the protein. Lactoferrin sequesters iron that is an essential growth factor for microorganisms (Otto et al. 1992). It disrupts bacterial type III secretion systems (Ochoa and Clearly, 2004) and destabilizes the outer membrane of Gram-negative bacteria resulting in the release of bacterial lipopolysaccharides (LPS; Ellison et al. 1988). However, this is not followed by an increase in proinflammatory cytokine secretion due to the property of bLF to bind LPS (Elass-Rochard et al. 1998) and the soluble LPS-receptor CD14 (Haversen et al. 2002). Since bLF administration proved to be a promising approach to clear EHEC from the ruminant gut, it is reasonable to assume that it can also be used to treat EHEC

infections in humans or prevent infections in persons at risk of exposure. Of note, bLF was already classified as a safe food supplement for human use (EFSA, 2012). However, its use in the context of human EHEC infections can only be considered if bLF, a natural anti-microbial agent, does not lead to an increased production and release of Stx, a phenomenon well known to occur in EHEC-infected patients treated with antibiotics (Zhang et al., 2000).

Two main types of Stx are produced by EHEC, namely Stx1 and Stx2. The *stx2* gene is frequently detected in human isolates, while the *stx1* gene is more commonly found in isolates of animal origin (Boerlin et al. 1999). Epidemiological data suggests that $Stx2^+$ strains are more likely to cause severe disease in humans than those producing only Stx1 or a combination of both types (Fuller et al. 2011). All Stxs possess an AB_5 molecular configuration in which the enzymatically active A subunit (molecular mass of approximately 32 kDa) is non-covalently associated with a pentamer of identical B fragments (molecular mass of 7.7 kDa, each), responsible for binding to the cellular receptor (Johannes & Römer 2010). The A subunit can be cleaved by bacterial proteases (Samuel & Gordon 1994) as well as by the host protease furin into the A2 fragment and the catalytically active A1, which then are still held together by a disulfide bond (Fagerquist & Sultan 2010).

To estimate the biological safety of bLF with respect to administration to EHEC-infected human patients, we analysed the effects of different bLF concentrations on: (1) Stx1 and Stx2 release by two EHEC strains of the most common EHEC-serotype O157:H7, (2) cytotoxicity of Stx2 for Vero cells, (3) cytotoxicity for Vero cells of treated EHEC, (4) cytotoxicity for Vero cells of adherent EHEC bacteria and finally (5) the stability of Stx2 taking into account the serine endopeptidase activity of bLF.

6.3 Methods

6.3.1 Cell culture.

Vero cells were grown in Minimum Essential Media (MEM; Thermo Fisher Scientific, Erembodegem, Belgium) supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, and 1% kanamycin. Subsequently, the cells were seeded into 96-well plates ($5x10^4$ cells/well in 100 µl culture medium) and allowed to form a monolayer during 24 hours.

6.3.2 Bovine lactoferrin.

Bovine lactoferrin (bLF) with 92% purity and 16% iron saturation (Ingredia Nutritional, Arras, France), derived from bovine milk, was used in this study.

6.3.3 Bacterial organisms.

Two bovine EHEC field isolates, strain 147 (O157:H7, $Stx2^+$) and strain 220 (O157:H7, $Stx1^+/Stx2^+$), and one *E. coli* isolate of human origin (strain NCTC12900; O157:H7, Stx^-; Dibb-Fuller et al. 2001) were used. The bacteria were grown overnight in Luria-Bertani (LB) broth with aeration (200 rpm) at 37°C, harvested by centrifugation (11,337 x g, 5 min), and re-suspended in medium to the desired concentration.

6.3.4 Effect of bLF on Stx release by EHEC strains.

All *E. coli* O157:H7 strains were grown overnight as described previously and subsequently diluted in LB broth to a concentration of $1x10^6$ CFU/ml. The LB broth was supplemented with 10, 1, 0.1 or 0 mg/ml bLF and cultures were placed in a shaking incubator with aeration (200 rpm, 37°C). Supernatant was collected after 6, 24, 48 and 72 hours of incubation and was analyzed using the Novitec® Verotoxin sandwich ELISA (HISS Diagnostics, Freiburg, Germany) and two in house ELISA assays. The commercial ELISA, which cannot distinguish between Stx1 and Stx2, was performed as described by the manufacturer. Briefly, diluted samples (1:3 in PBS, pH 7.4) were added in duplicate to ELISA plates pre-coated with polyclonal Stx antibodies and incubated (30 min). After washing, reagent 1, containing a monoclonal anti-Stx antibody cocktail was added and the plate was incubated at room temperature (30 min). The plates were washed and reagent 2, containing an anti-mouse horseradish peroxidase conjugated antibody, was added and the plates were incubated for another 30 min at room temperature. After a washing and subsequent rinsing step with distilled water, the substrate solution, containing chromogen tetramethylbenzidine (TMB; Life Technologies GmbH, Darmstadt, Germany) and peroxide was added. The absorbance was measured at 450 nm. The values were presented as Standard% (Std%) values with standard controls included in the kit used as standard. The background signal, i.e. the signal generated by supernatants of the NCTC12900 strain (Stx^-), was always below 2 Std%. Supernatants sampled after 6 hours incubation were also tested using two in-house ELISA assays for

detection of different Stx1 and Stx2 variants modified from Segura-Alvarez et al. (2003). Briefly, microtiter plates were coated overnight at 4°C with hydatid cyst fluid from *Echinococcus granulosus* infected sheep in sodium carbonate buffer (pH 9.5). Thereafter, the plates were washed with PBS-Tween (0.05% Tween®20 in PBS, pH 7.4) and non-specific binding sites were blocked using blocking buffer containing 1% milk powder in sodium carbonate buffer (pH 9.5) for 1 h at 37°C. The plates were washed again with PBS-Tween and incubated for 2 hours at 37°C with samples (duplicates, 1:3 diluted in PBS-Tween). After washing with PBS-Tween, horseradish peroxidase conjugated antibodies against either Stx1 (clone VT109/4-E9b, α-Stx1 B subunit; SIFIN Institut für Immunpräparate & Nährmedien GmbH, Berlin, Germany) or Stx2 variants (clone VT135/6-B9, α-Stx2 A subunit; SIFIN) were added (1:1000 diluted in PBS-Tween; 1 h, 37°C). Plates were washed with PBS-Tween and the TMB chromogen solution was added (15 min). Finally, stop solution (0.5 M H_2SO_4) was added. The optical density was measured at test (450 nm) and reference wavelengths (550 nm) and the OD values for all samples calculated as difference between test and reference value. The real value was calculated as mean OD value of the sample minus mean OD value of blank. Standardization was performed using standard controls and values were presented as Std% using recombinant Stx1 toxoid (1:675 diluted in PBS-Tween;(Kerner et al. 2015)) and recombinant Stx2e toxoid (1:18225 diluted in PBS-Tween; kindly provided by Prof. Dr. R. Bauerfeind, Justus-Liebig-University Gießen, Germany) either as positive or as negative control in the in-house ELISA assays.

6.3.5 Bovine lactoferrin cytotoxicity assay.

To check the putative cytotoxic effect of bLF, Vero cells were seeded in 96-well plates (Greiner Sigma-Aldrich, Diegem, Belgium) at a concentration of 5.10^4 cells/well in 100 µl culture medium and, after overnight incubation at 37°C and 5% CO_2, exposed to final concentrations of 100, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 and 0 mg/ml of bLF in 200 µl culture medium. After 6, 24, 48 and 72 hours cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, measuring mitochondrial activity (Mosmann 1983). Twenty microliter MTT (5 mg/ml, Sigma-Aldrich, Diegem, Belgium) in Hanks' balanced salt solution (Thermo Fisher Scientific, Erembodegem, Belgium) was added to each well and after 4 h of incubation at 37°C and 5% CO_2, the MTT solution was removed and 200 µl/well DMSO in ethanol

(1/1 v/v) was added. The plates were shaken for 15 min at 450 rpm to dissolve the formazan crystals and subsequently analyzed spectrophotometrically at a test wavelength of 590 nm (OD1) and a reference wavelength of 635 nm (OD2). Final optical densities obtained from formazan formation were presented as OD1 minus OD2. The experiments were conducted in triplicates and each experiment was repeated three times.

6.3.6 Influence of bLF on toxin release by freshly inoculated EHEC bacteria or by EHEC bacteria attached to Vero cells.

The influence of bLF on Stx release by freshly inoculated EHEC bacteria was examined as follows: Vero cells were seeded in 96-well plates as described above. The following day, bLF (10, 1, 0.1 and 0 mg/ml, respectively) was added in 100 µl cell medium (without antibiotics) together with the bacteria (10^6 CFU/well) or partially purified Stx2 (5 ng/ml; Toxin Technology Inc, Sarasota, USA). Cells incubated with bacteria only served as controls. After 24, 48, and 72 hours, bacteria and cell debris was removed by thorough washing with culture medium and the metabolic activity of the remaining Vero cells, which is indirectly linked to the Stx release, was measured by MTT assay as described above. Data was calculated as percent metabolic activity relative to cells incubated in cell culture medium only (values set to 100%) and blank wells (values set to 0%).

Since EHEC colonize the gut and bLF has to prevent cytotoxic effects of colonizing bacteria, a similar experimental set up was used to test the influence of bLF on EHEC bacteria adhering to Vero cells (Tahamtan et al. 2011). Hereto, the bacterial strains (10^6 CFU/well) were allowed to attached for 6 h, after which non-adherent bacteria were removed and bLF (10, 1, 0.1 and 0 mg/ml, respectively) was added to different wells. Cells incubated with bacteria only served as controls. Again, Stx release was measured indirectly by performing an MTT assay at 24, 48, and 72 hours after washing off of bacteria and cell debris. Data was calculated as percent metabolic activity as described above. All these experiments were conducted in triplicates and each experiment was repeated three times.

6.3.7 Effect of bLF on Stx2.

To determine if bLF cleaves Stx2, SDS-PAGE and Western blotting were used. Ten µg/ml of Stx2 were incubated overnight with 1000, 100, 10, 1 or 0 µg/ml bLF in PBS

(pH 7.4, 37°C), while gently shaking. Then the samples were heated (5 min, 65°C) or treated with 0.01 M β-mercaptoethanol (5 min, 95°C) to reduce disulfide bonds, and then run on a 12% SDS-PAGE (45 min, 200 V). Western blotting was performed (350 mA, 90 min) and blots were developed with monoclonal mouse anti-Stx2 A-subunit antibody (1:500; Santa Cruz Biotechnology Inc, Dallas, Texas, USA) or monoclonal mouse anti-Stx2 B-subunit antibody (1:1000; clone 3C10E10H10, Abcam, Cambridge, UK) and rabbit anti-mouse HRP antibody (1:1000; Dako, Heverlee, Belgium). The Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Erembodegem, Belgium) was used for detection.

6.3.8 Statistical analysis.

GraphPad Prism was used for statistical analysis and graphic design of figures. Data on the influence of bLF on Stx production were analyzed using Tukey's multiple comparisons test to identify statistic significant differences ($p<0.05$) between groups at each time point. Results of the survival rate with different concentrations of bLF were analyzed using repeated measures one-way ANOVA for multiple comparisons after confirmation of Gaussian distribution. Significant differences between groups ($p<0.05$) were determined using a *post hoc* test with Bonferroni correction. Data on the metabolic activity and colonization effect was analyzed using a two-way ANOVA with multiple comparisons. Differences were considered significant if $p<0.05$.

6.4 Results

6.4.1 Bovine LF concentration-dependent decrease of Stx in bacterial culture supernatants.

The amount of Stx in bacterial culture supernatant is the result of production and secretion by the bacterial strains, but also Stx destruction and/or binding by bLF might occur. The Stx concentrations in supernatants of bacterial cultures after 6, 24, 48 and 72 hours incubation with 10, 1 and 0.1 mg/ml bLF or without bLF (control) are presented in Figure 6.1. Ten mg/ml bLF showed the strongest reduction in released Stx. As early as 6 h post inoculation of cultures with strain 147 ($Stx2^+$), presence of 10 mg/ml bLF resulted in significant and sustained reduction of Stx concentrations compared to the control whereas cultures of strain 220 ($Stx1^+/Stx2^+$) only showed this effect beginning 24 hours post inoculation. One tenth of the bLF concentration (1 mg/ml) exhibited similar

effects on strain 147 ($Stx2^+$), but reduced amounts of cell-free Stx in cultures of strain 220 ($Stx1^+/Stx2^+$) at 24 hours only. The effect of 0.1 mg bLF was even less pronounced but became significant for strain 147 ($Stx2^+$) at 24 hours. Using ELISA for specific detection of Stx1 and Stx2 variants, no Stx could be detected in supernatants of strain NCTC12900 (Stx^-; data not shown). Supernatants of strain 220 ($Stx1^+/Stx2^+$) sampled at 6 hours contained higher Stx1 concentrations with 10 mg/ml bLF than with lower bLF doses suggesting increased release (bacterial lysis) or production of Stx1 (Figure 6.2). However, 1 and 0.1 mg/ml bLF tended to dose-dependently decrease cell-free Stx1. This effect was more pronounced for Stx2 as its concentrations were below the detection limit when bacteria were cultured in the presence of 10 and 1 mg/ml bLF. Strain 147 ($Stx2^+$) did not show a release of Stx2 into the supernatant when tested by the specific ELISA (data not shown). Subsequent PCR tests (performed at the Scientific Institute of Public Health, Brussels, Belgium) revealed that strain 147 encodes for the Stx2c variant, which is not detected by the monoclonal antibody used here and known to recognize Stx2a but not Stx2c, d, and g (N. Deininger, S. Barth and L. Geue, personal communication).

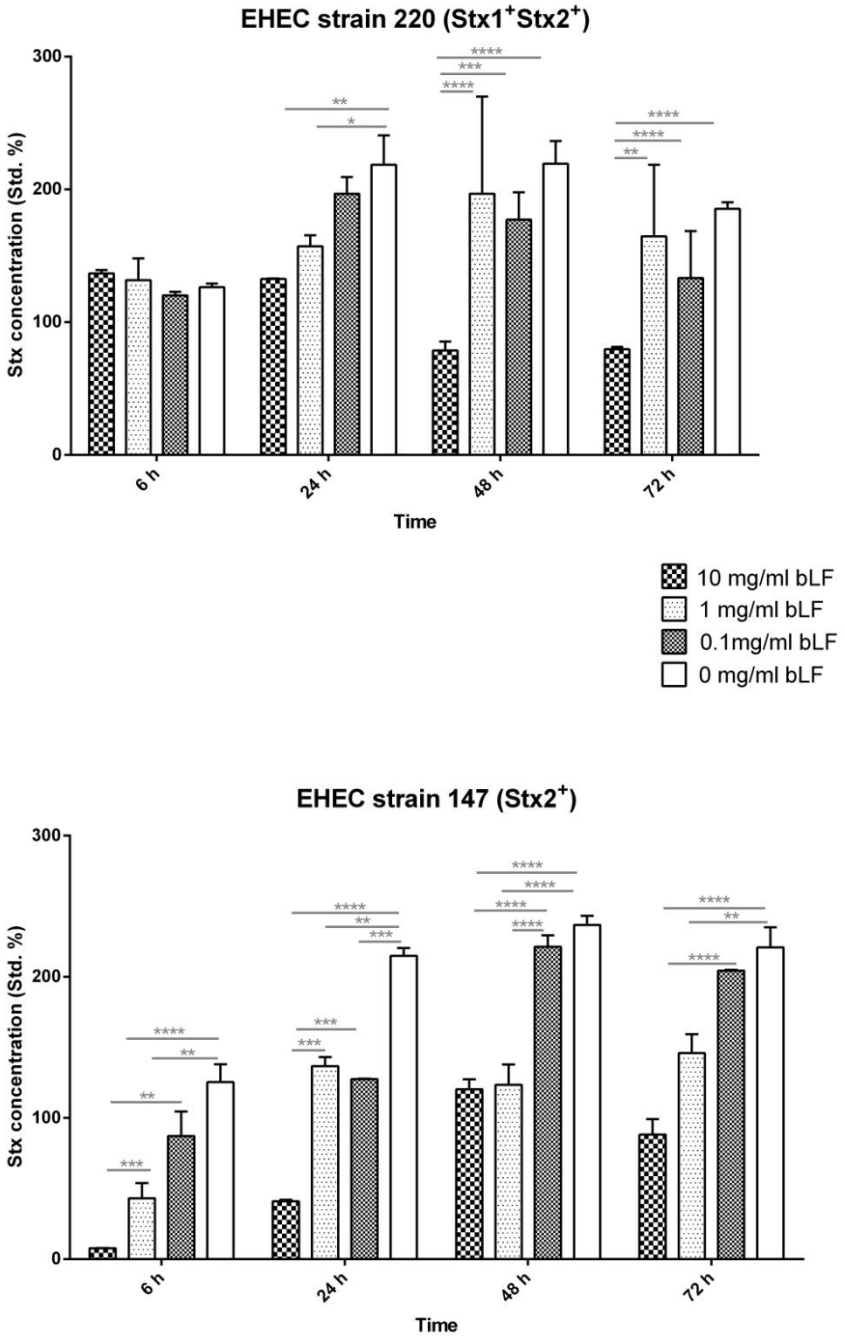

Figure 6.1: Standard % values of Stx concentration in supernatant of bacterial broth cultures after incubation with different bLF concentrations determined by a commercial Stx-specific ELISA (Verotoxin ELISA). Data is presented as percent standard relative to standards included in the kit. Error bars indicate standard deviations. Significant differences were indicated with *(*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$).

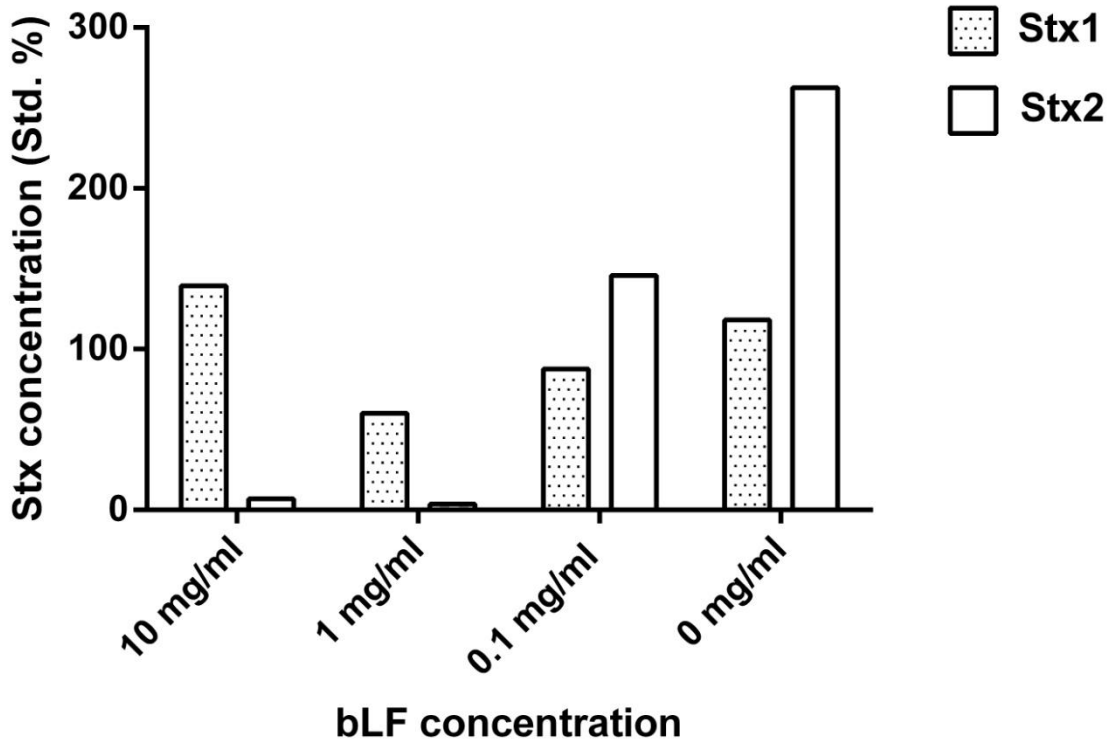

Figure 6.2: Standard % values for Stx1 and 2 concentrations in supernatant of strain 220 ($Stx1^+/Stx2^+$) cultured for 6 hours in the presence of different concentrations of bLF (results of in-house ELISA assays). Data is presented as percent standard relative to rStx1 toxoid and rStx2e toxoid used either as positive or and as negative control, respectively. Results are means of technical duplicates after standardization.

6.4.2 High concentrations of bLF exert cytotoxicity on Vero cells.

Bovine LF significantly reduced the metabolic activity (%) of Vero cell cultures from 48 hours incubation onwards (Figure 6.3; other time points not shown). This was dose-dependent as 100 mg/ml reduced the metabolic activity to 41.4%, whereas 50 mg/ml resulted in 64.6% activity. The reduction was significantly different ($p<0.05$) from control cultures (0 mg/ml bLF) for concentrations of 50 mg/ml bLF and above. The highest bLF concentration of lactoferrin considered non-toxic for Vero cell cultures was 10 mg/ml.

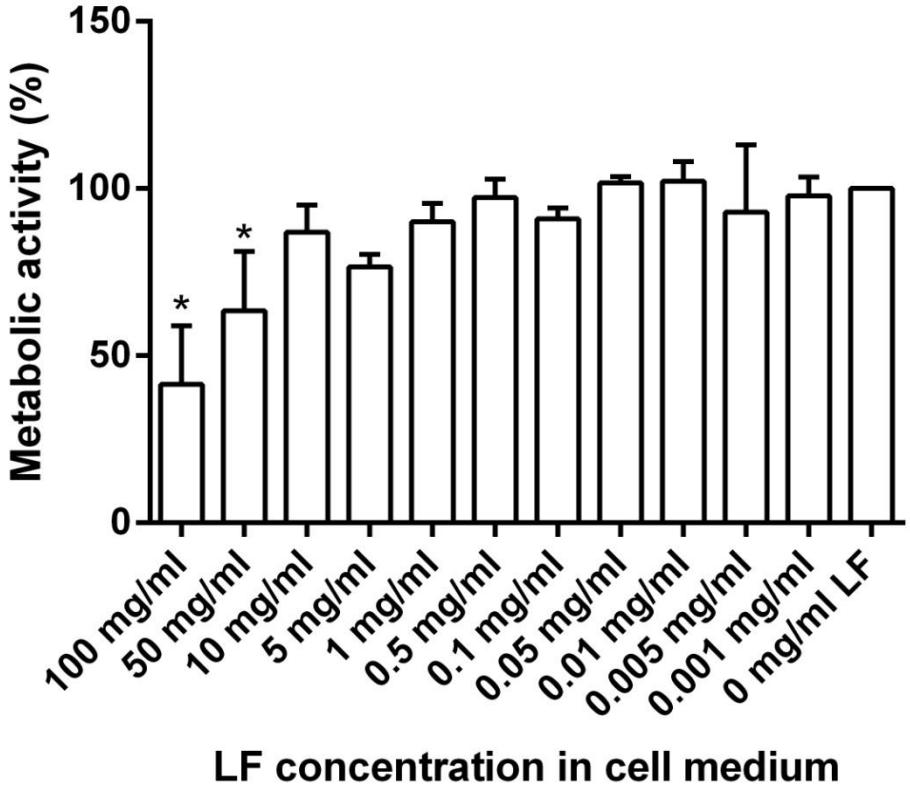

Figure 6.3: Mean metabolic activity of Vero cells in cell medium supplemented with different concentrations of bLF. Data is presented as percent metabolic activity relative to cells incubated in cell culture medium only (values set to 100%) and blank wells (values set to 0%). Results are means of 3 biological replicates with n=3. Error bars indicate standard deviations. Significant differences compared to the control cultures without bLF were indicated with *.

6.4.3 Bovine LF can reduce verocytotoxicity of EHEC when co-cultured with Vero cells.

In the first set-up Vero cells were grown to confluence for 24 hours whereafter different concentrations of bLF together with the O157:H7 *E. coli* strains were added. The metabolic activity of the Vero cells after different times of incubation with 10^6 CFU/ml of bacteria with or without bLF is presented in Figure 6.4. Although 0.1 mg/ml bLF had limited effects on the amount of cell-free Stx in bacterial cultures, it partially protected Vero cells co-cultured with each of the EHEC strains from cytotoxic effects. This effect was significant for strain 220 ($Stx1^+Stx2^+$) after 24 hours of incubation and for strain 147 ($Stx2^+$) after 48 and 72 hours of incubation. Presence of 1 mg/ml bLF also induced a significantly higher metabolic activity at 24 hours of incubation with strain 220 ($Stx1^+Stx2^+$), but not with strain 147 ($Stx2^+$), nor at any other time point. Ten mg/ml bLF even seems to increase cytotoxicity for both EHEC strains which was particularly evident after 24 hours.

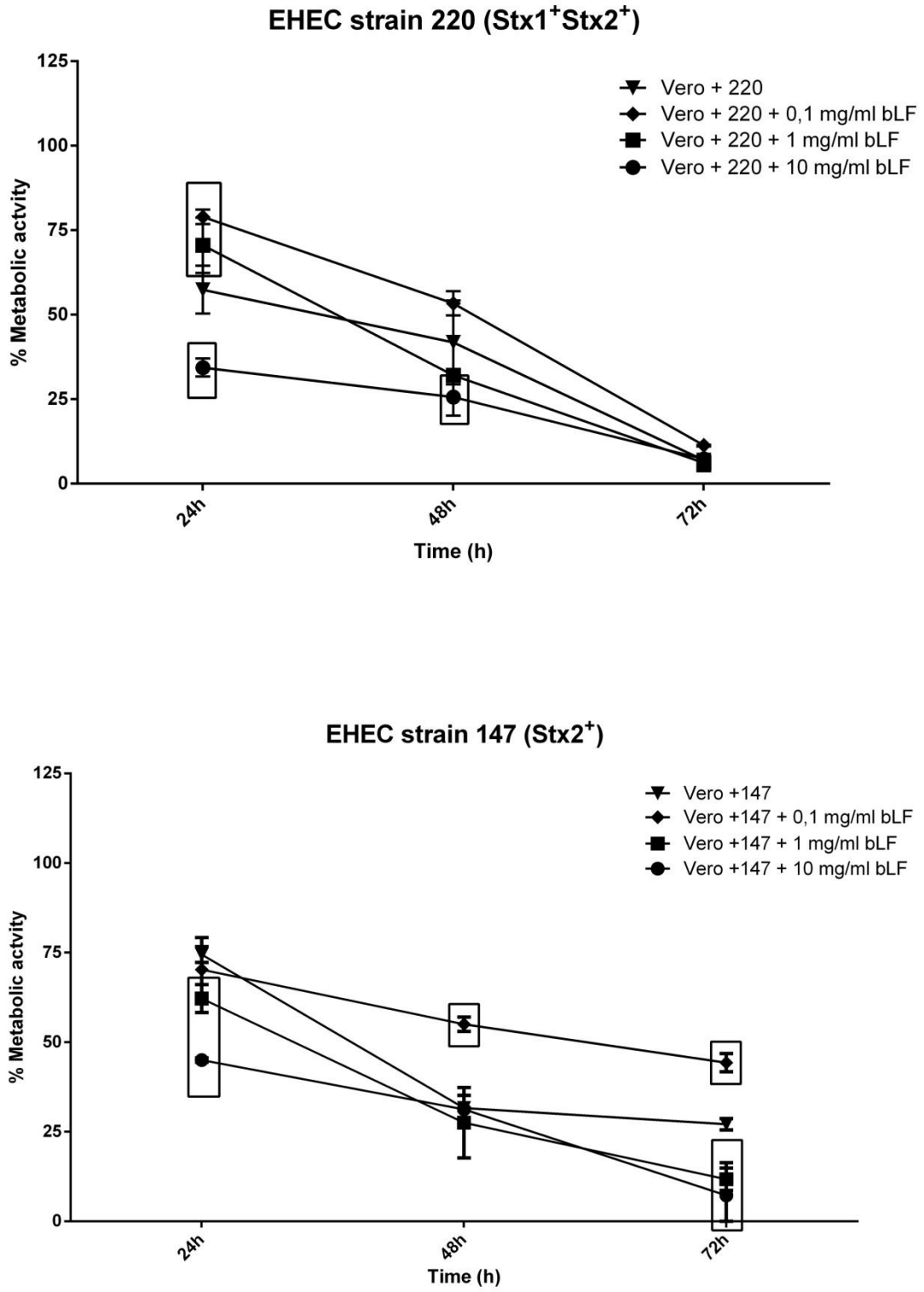

Figure 6.4: Metabolic activity of Vero cells following incubation with 10, 1 or 0.1 mg/ml bLF in combination with EHEC strain 220 (Stx1$^+$Stx2$^+$) and strain 147 (Stx2$^+$). Data is presented as percent metabolic activity relative to cells incubated in cell culture medium only (values set to 100%) and blank wells (values set to 0%). Results are means of 3 biological replicates with n=3. Error bars indicate standard deviations. The data points in the rectangles are significantly different from the controls.

When Vero cells were incubated with purified Stx2, 0.1 mg/ml bLF did not mitigate verocytotoxic effects. An increased toxicity was observed for 1 and 10 mg/ml bLF although this effect was less pronounced for 10 mg/ml (data not shown).
Vero cells grown in presence of strain NCTC12900 (Stx^-) showed a impaired metabolic activity when incubated with the three concentrations of bLF and this was dose dependent after 48 and 72 hours of incubation (data not shown).

To mimic potential effects of bLF during intestinal EHEC colonization, an experiment was set-up in which bacteria were allowed to adhere for 6 hours to Vero cells, whereafter non-adhering bacteria were washed away before bLF was added (Figure 6.5). Interestingly, this experimental set-up led to a clear reduction of cytotoxicity by different concentrations of bLF until 48 hours of incubation. Especially for strain 220 ($Stx1^+Stx2^+$) a significantly higher metabolic activity was observed at 24 hours for 10, 1 and 0.1 mg/ml bLF and at 48 hours for 10 and 1 mg/ml bLF. This effect was less pronounced for strain 147 ($Stx2^+$). Here, a significantly higher metabolic activity was seen at 48 hours for all three bLF concentrations.

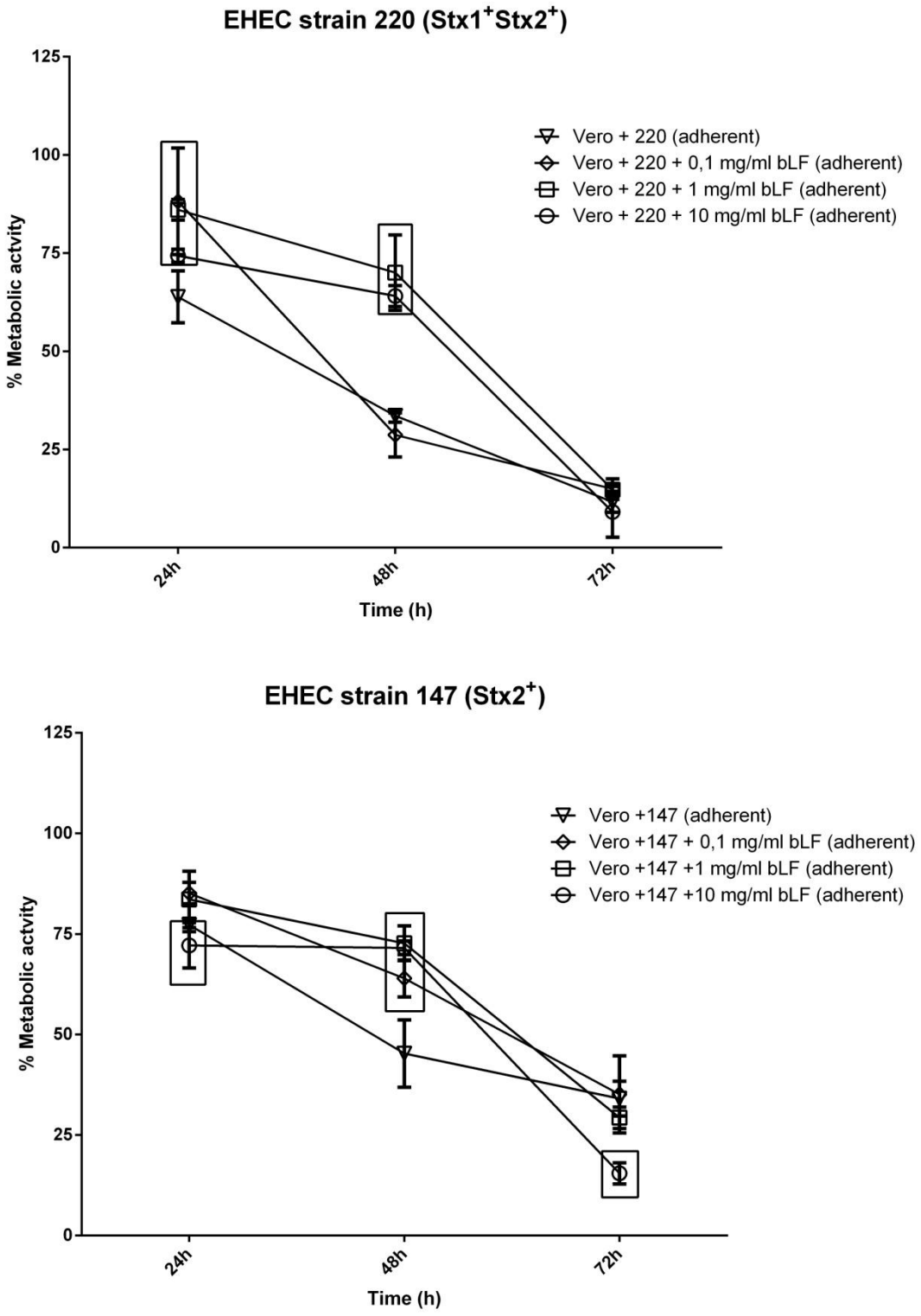

Figure 6.5: Metabolic activity of Vero cells colonized with the EHEC strains and incubated with different concentrations of bLF. Data is presented as percent metabolic activity relative to cells incubated in cell culture medium only (values set to 100%) and blank wells (values set to 0%). Results are means of 3 biological replicates with n=3. Error bars indicate standard deviations. The data points in the rectangles are significantly different from the controls.

6.4.4 Proteolytic effect of bLF on Stx2.

To investigate if the protease activity of bLF is able to degrade Stx2, we performed SDS-PAGE and Western blotting (Figure 6.6). While the A subunit can be detected at a molecular weight of almost 31.4 kDa in non-reducing conditions, it mainly presents as a 27 kDa band under reducing conditions indicating the cleavage of the disulfide bound allowing the A1- and A2-fragment to migrate independently with only the A1-fragment being visualized by the monoclonal antibody (upper part of the figure). The presence of increasing concentrations of bLF led to greater band intensities of the A-subunit under both conditions pointing to some stabilizing effect of bLF under these experimental conditions. By stark contrast, incubation of Stx2 with bLF at all concentrations applied drastically reduced the band intensities for B-subunit detection (lower part of the figure) under reducing and even totally abolished B-subunit detection under non-reducing conditions.

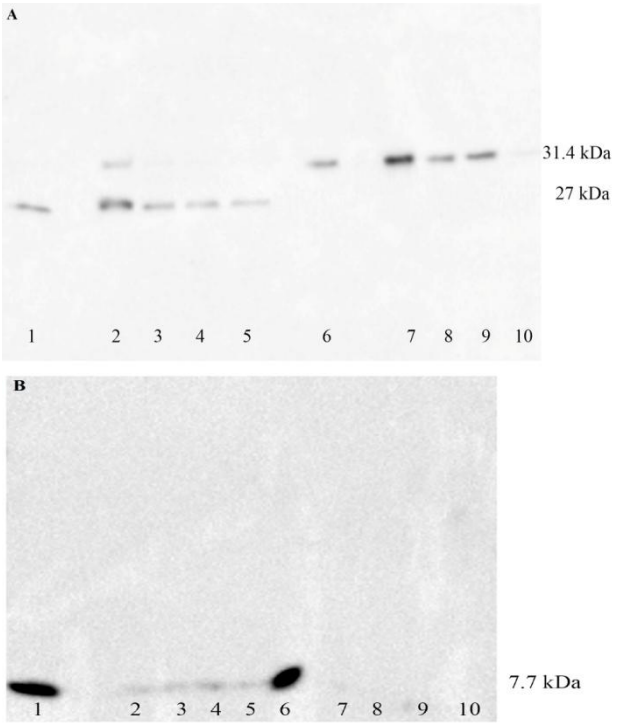

Figure 6.6: Stability of Stx2 in the presence and absence of bLF.
Partially purified Stx2 was incubated for 16 hours at 37°C and sizes and relative amounts of Stx2 subunits were assessed by Western blotting after gel electrophoresis under reducing (left lanes) and non-reducing conditions (right lanes). In the upper Western blot (A) detection with anti-Stx2-A subunit-antibody, in the lower (B) with anti-Stx2-B subunit-antibody was conducted. Molecular weights (kilodalton) are indicated on the right. Lane 1: Stx2 + β-mercaptoethanol; Lane 2: Stx2 + 1 mg/ml bLF + β-mercaptoethanol; Lane 3: Stx2 + 100 µg/ml bLF + β-mercaptoethanol; Lane 4: Stx2 + 10 µg/ml bLF + β-mercaptoethanol; Lane 5: Stx2 + 1 µg/ml bLF + β-mercaptoethanol; Lane 6: Stx2; Lane 7: Stx2 + 1 mg/ml bLF; Lane 8: Stx2 + 100 µg/ml bLF; Lane 9: Stx2 + 10 µg/ml bLF; Lane 10: Stx2 + 1 µg/ml.

6.5 Discussion

To our knowledge, this is the first *in vitro* study investigating the effect of bLF on the release and bioactivity of Stx by EHEC. In the past, other *in vitro* and clinical studies have focused on the release of Stx and outcome of patients when antibiotics were used as a treatment. *In vitro* work indicated that both sub-inhibitory and sub-lethal concentrations of antibiotics (polymyxin B, trimethoprime/sulphamethoxazole, ciprofloxacin, cefixime, and tetracycline) increase the release of toxins in the supernatant of EHEC O157:H7 cultures (Karmali et al. 1985; Karch et al. 1986; Tittor 2003; Walterspiel et al. 1992). An *in vitro* study on a common EHEC O157:H7 strain and the O104:H4 German outbreak strain (2011) showed that therapeutic concentrations of the antibiotics ciprofloxacin, meropenem, fosfomycin, gentamycin, rifampicin, and chloramphenicol did not cause release of Stx from the O104:H4 strain but did from the O157:H7 strain (Corogeanu et al.

2012). Several clinical studies have evaluated the possible benefit or harm of antibiotics on the clinical course of *E. coli* O157:H7 colitis, mainly by estimating their effect on the duration of the illness, diarrhoea and/or bloody diarrhoea, on the risk for development of HUS or thrombotic thrombocytopenic purpura and on the death rate. Some studies associate the administration of antibiotics with higher risk for HUS as well as with longer duration of diarrhoea and/or bloody diarrhoea. Patients treated with trimethoprim/sulphamethoxazole tended to have longer duration of diarrhoea and bloody diarrhoea and were more likely to develop HUS than patients who did not receive an antibiotic, although this negative effect was not seen for ampicillin treatment (Pavia et al. 1990; Ostroff et al. 1989). Antibiotics affect the release of Stx, but do not cause degradation of the toxin. In this study we provide evidence that the receptor binding subunit of Stx2 is degraded by bLF. The effect on the enzymatic activity of the degraded toxin has to be investigated, but degradation of the B-subunit is likely to prevent binding of the toxin to the cellular receptor and subsequent internalization, both being prerequisites for the major cytotoxic effects exhibited by Shiga toxins to occur.

In the present study, we used two Stx ELISA assays to quantify Stx. The commercial ELISA is a sandwich ELISA using polyclonal anti-Stx 1 and 2 antibodies to capture the antigen in the sample. After capturing the antigen, an anti-Stx antibody cocktail is added recognizing the bound antigen. The in-house ELISA can distinguish between Stx1 and Stx2. Two O157:H7 EHEC strains showed a bLF dose-dependent reduction in Stx release in the supernatant which was significant for 10 mg/ml bLF, the highest concentration tested in this part of the study. This might be the result of the bacteriostatic effect of bLF on the bacteria. In a previous study 1 mg/ml exerted a similar bacteriostatic effect than 10 mg/ml in our study (Atef Yekta et al., 2010). The sensitivity for bLF is likely strain dependent and we suggest that other factors are also influenced by bLF. This is supported by the results of the in-house ELISA assays for strain 220 ($Stx1^+Stx2^+$). Indeed, 6 hours incubation with 10 mg/ml bLF did not reduce the concentration of Stx1 in the culture medium, whereas Stx2 became low to undetectable.

Unfortunately, the in-house ELISA assay was unable to detect Stx2 encoded for in strain 147 ($Stx2^+$), because that strain harbours the Stx2 c gene that differs from the subtype produced by strain 220 ($Stx1^+Stx2^+$), Stx2a. Nevertheless, the results of the

commercial Stx-specific ELISA showed similar results for both strains supporting the hypothesis that bLF can reduce Stx2 production and release.

To confirm that reduction in the amounts of cell-free toxins translates into reduction in cytotoxic effects under conditions similar to EHEC colonization of an intestinal mucosa, the effect of bLF had to be tested in a cell culture system which allowed for measuring toxicity of Stx. An *in vitro* Vero cell model was used because of the sensitivity of this cell line for Stx (Tahamtan et al. 2011). Incubation of Vero cells with different bLF concentrations, suggested that concentrations of 10 mg/ml and less retained a stable metabolic activity of the cells. However, once used in combination with bacterial inoculation of the Vero cells, the 10 mg/ml bLF was seen to reduce the metabolic activity. In contrast, 0.1 mg/ml bLF had a positive effect on metabolic activity of cells incubated with toxin producing strains for at least 48 hours, even though it did not have a consistent effect on the toxin concentration in bacterial culture supernatants. One mg/ml bLF significantly reduced toxin concentrations at 24 hours incubation as well as it significantly reduced cytotoxicity, suggesting that a daily administration by a bolus using a suitable dose might be a valuable strategy for treatment of infected subjects. EHEC deploy a type III secretion system to colonize the intestinal mucosa. Previously we demonstrated that bLF can reduce attachment of a non-toxin producing O157:H7 strain to Caco-2 cells (Yekta et al. 2010). Furthermore, treatment with bLF can completely clear rectal colonization with strain NCTC (Stx^-) used in the present study in calves *in vivo* (Chapter IV). However, in case of colonization with EHEC, toxins might be released in close proximity with the mucosa being unavailable for interaction with bLF.

Since results pointed to an effect of bLF on the bioactivity mainly of Stx2, we examined if the protein could cleave the toxin. The presence of increasing concentrations of bLF led to greater band intensities for detection of the A-subunit suggesting some stabilizing effect of bLF under these experimental conditions. As Stx2 was obtained in a partially purified form, making it particularly sensitive to residual protease activities by contaminating bacterial products in the solution (C. Menge, personal observation), addition of hundred-fold excess of protein by addition of 1000 µg/ml of bLF to 10 µg of Stx2 may have prevented unspecific proteolytic cleavage of the A-subunit. The results of the respective experiment clearly indicated however, that incubation of Stx2 with bLF resulted in an effective removal of the B-subunit that could no longer be detected.

Destruction of the receptor binding B subunit by bLF may have protective effects like B-subunit specific antibodies that efficiently protect cells from the detrimental effects of Shiga toxins (Stamm et al. 2002). Destruction of the receptor binding B subunit by bLF may have similar effects. Although the effects for Stx1 seem to be less pronounced, it would be interesting to also analyze the possible proteolytic effect of bLF on Stx1.

6.6 Conclusions

The results presented here are of major importance for the application of bLF to reduce EHEC-carriage in cattle. Indeed, there is evidence that *E. coli* O157:H7 Stx can actively suppress cellular immune responses in cattle what would enable long-term colonization (Hoffman et al. 2006). If further *in vivo* experiments with cattle can confirm a reduction or complete clearance of *E. coli* O157:H7 at farm level, bLF can be systematically applied on infected farms on fattened animals before slaughter. Results are also encouraging for considering the use of bLF as treatment of EHEC infections in humans. In 2012, bLF has been approved by EFSA to be safe for the use as an ingredient for food supplements, infant and follow-on formulae, dietetic food for special medical purposes and sports nutrition, and for a variety of foods (Efsa, 2012). The therapeutic treatment of infected patients with bLF at non-toxic concentrations which are sufficient to limit EHEC growth and Stx production and release, at the same time degrading the B-subunit of still released Stx2, might be a promising strategy worthwhile further investigation.

6.7 Acknowledgements

We thank M. J. Woodward for providing *E. coli* strain NCTC12900 and R. Bauerfeind for providing rStx2e. This research was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment (RF10/6233).

Chapter VII: General discussion & future perspectives

The objective of this thesis was to gain insights in modulation of the local immune response by *E. coli* O157:H7 in cattle and to analyse whether and how bovine lactoferrin (bLF) could influence this immune response. For this reason, the setup of this thesis was divided in four main goals: (1) understanding factors promoting *E. coli* O157:H7 colonization in cattle by studying immune responses; (2) investigating modulation of systemic immune responses by bLF and reduction of an *E. coli* O157:H7 infection in cattle with bLF; (3) determining the effect of different *E. coli* strains and bLF or Shiga toxin (Stx) on the release of proinflammatory cytokines; (4) exploring possible effects of bLF against Stx-positive EHEC strains.

First, the effect of an *E. coli* O157:H7 infection on the bovine host was characterized by investigating the gene expression in the recto-anal junction (RAJ) and the ileal Peyer's patches (I+PP) in calves that were infected once or twice with *E. coli* O157:H7 (Stx^-) compared to calves that were not infected.

Many studies have described the persistence of *E. coli* O157:H7 in ruminants like cattle and sheep, in observational and experimental settings (Rahn et al. 1997; Cornick et al. 2000; Lim et al. 2007; Aktan et al. 2007; Vande Walle et al. 2011; Joris et al. 2013b). Both *in vitro* and *in vivo* studies in ruminants and case studies in humans have focused on many different aspects of the infection with *E. coli* O157:H7, but none of them described the effects of this infection on a genome-wide scale at two important sites in the gastro-intestinal tractus, like we did in Chapter III. Previous research has aimed at unraveling effects of an EHEC infection on individual cell types. Studies using primary human colonic epithelial cells and Caco-2 cells showed that H7 flagellin recognition by TLR5 is the major factor inducing IL-8 production upon infection (Miyamoto et al. 2006; Fraser-Pitt et al. 2011). This is found to be a downstream effect as a result of the activation of p38 and ERK MAP kinases and the nuclear translocation of the transcription factor NF-κB (Berin et al. 2002). However, recent studies investigating the bovine TLR5 indicate that cattle express a functional TLR5 with different flagellin sensing qualities compared to human TLR5, and that other potentially intracellular expressed receptors may play a more important role in the bovine system to detect flagellin (Metcalfe et al. 2014, Osvaldova et al. 2014). Intestinal epithelial cells like the polarized T84 and HT-29 and non-polarized HeLa cells showed a significant increase in IL-8 and TNF production during infection with type IV pilus-expressing EHEC-strains (Ledesma et al. 2010).

Infection of human T84 and HEp-2 epithelial cells infected with EHEC O157:H7 and stimulation with IFN-γ revealed elimination of the IFN-γ-induced Stat1-tyrosine phosphorylation and disruption of the inducible protein expression of the Stat1-dependent gene interferon regulatory factor 1. This results in an impaired host response as Stat1 cannot modulate the transcription of responsive genes (Ceponis et al. 2003). Models using the *C. rodentium* infection in mice found a strong Th1/Th17 response in the colon of infected animals, characterized by an increased expression of IL-1β, TNF, IL-12, IFN-γ, IL-17 and IL-22 (Higgins et al. 1999; Geddes et al. 2011; Zheng et al. 2008). Furthermore, strain-specific immunomodulation was described by Corbishley et al. (2014) for $CD4^+$ T-cells, NK-cells and $CD8^+$ and γδ T-cells.

The RNA-Seq technology, which was used in our gene expression study, allows RNA analysis through cDNA sequencing at massive scale. While initial transcriptomics techniques largely relied on hybridization-based microarray technologies offering a limited ability to fully describe and quantify the diverse RNA molecules which are expressed from genomes over wide ranges of levels. Furthermore, RNA-Seq allows to work with minute RNA quantities and to provide quantification of RNA samples from even single cells. This gives us the opportunity to define complex biological networks in a wide range of biological specimens. With these networks in hand, data-driven RNA network models of cells and tissues can be used in an attempt to fully understand the biological pathways that are active in various physiological conditions (Ozsolak & Milos 2011).

However, this advantage can also be seen as a drawback since it is very hard to interpret data of a plethora of genes which seem to be affected. In addition, there are experimental considerations that have to be taken into account when performing this technique. The expression of genes is time and cell dependent, so the results compiled from this technique will lead to information that is only valuable for one time point, as these samples were taken at the time of euthanasia. A time course experiment starting from a few hours after ingestion, during colonization, until potential clearing of the bacterium could give extra valuable information. The cell dependency of the transcripts is important as only small amounts of RNA will be further processed and analyzed with this technique. A major point of concern is the subjectivity of the analysis, as the results can vary due to the multitude of algorithms available. Most of the approaches are correct,

but have to be tailored to the needs of the investigators in order to better capture the desired effect. This variability in methods, although at smaller scale, is also present in other RNA profiling approaches where reagents, personnel and techniques can lead to similar, although statistically different, results. Taking this into account, the downstream interpretation of data must show biological, clinical and regulatory functions which allows for drawing meaningful conclusions.

The tissue specificity of this technique allowed us to discover that the gene-transcripts in the Peyer's patches are far more affected by an infection with *E. coli* O157:H7 than the gene-transcripts of the rectum. This was surprising because the terminal rectum is the predilection site of the bacterium, but this leaves space for two hypotheses. A first hypothesis could be that after oral intake, the bacteria pass through the gastro-intestinal tract and adhere –shortly- to the ileum and/or close to the Peyer's patches while influencing the local gene expression. A second hypothesis could be that the bacteria colonize the lymphoid follicle-dense mucosa in the rectum (Naylor et al. 2003) while secreting specific factors that are taken up by immune cells or into the numerous blood vessels and these factors are transported to the Peyer's patches where they influence gene expression. The first hypothesis is plausible as fecal shedding gradually disappears after clearing of the rectal mucosa. Rybarczyk et al. (2015) has suggested that this might be due to a temporary presence or colonization of the bacteria of more cranial intestinal mucosal sites, which could be the Peyer's patches. Furthermore, colonization of these sites is also supported by cases of diarrhea in EHEC-infected calves. Here, ileal and colonic mucosae are also colonized with EHEC (Dean-Nystrom et al. 1997) and some viable bacteria are even translocated to the mesenteric lymph nodes (Cray & Moon 1995). The second hypothesis is possible, but we could not find any report, recent or older, describing a direct link between the highly vasculated rectal mucosal tissue and the ileal Peyer's patches in cattle. In humans it has been shown that the rectal route bypasses around two thirds of the first-pass metabolism as the rectum's venous drainage is two thirds systemic (middle and inferior rectal vein) and one third portal (superior rectal vein). As a result, compounds that are applied rectally can easily reach the circulatory system (de Boer et al. 1982). Furthermore, a study in mice confirmed that intrarectal immunization induces homing of lymphocytes by $\alpha_4\beta_7$ integrin, which enables lymphocytes to migrate from the rectum into the small intestine (Agnello et al. 2013). A similar transport of factors or cells might be possible in cattle, but this

needs to be confirmed in experimental studies. Despite this, both hypotheses cannot explain why the effects on gene expression levels were only detected in the ileum and not in the RAJ, leaving a hiatus worth further investigating.

The finding that a first infection has more effect on the gene transcription then a secondary exposure affirms our observations that a first infection primes the immune system making a second colonization lasting longer. In our study, we reported the results of an infection with a Stx-negative strain. Since Stxs have shown to provoke many effects on cellular level as described in Chapter 2.3, it might be useful to perform the experiment with different Stx-positive strains as strain-specific effects are conceivable (Corbishley et al. 2014).

In our cytokine study (Chapter V), we described the effect of three different *E. coli* O157:H7 strains which were Stx^-, $Stx2^+$ or $Stx1^+Stx2^+$, as we analysed the release of the proinflammatory cytokines IL-6 and TNF-α and the chemokine CXCL8 by rectal epithelial cells and explants upon *E. coli* O157:H7 infection. Whereas, we could not observe an effect on these cytokines in our RNA-Seq study (Chapter III), we found clear effects on cells and tissue explants. This difference might be explained by the different time frames after infection/inoculation that were used in the different experiments. In the RNA-Seq study, samples were collected 14 days after a first or re-infection and mRNA was analysed. It is well know that the mRNA expression of cytokines is tightly regulated, as a failure to degrade proinflammatory cytokine transcripts might lead to chronic inflammation (Rattenberger, 2012). Moreover, the half-life of cytokine mRNA typically is in the range of hours rather than days (Iwasaki 2011, Villarete 1996). This knowledge, combined with the immune suppression by different (non-)LEE-encoded effectors upon attachment and colonization, might explain why we could not detect effects on proinflammatory cytokines 14 days after infection. Measurement of protein levels in the supernatants after six hours of incubation as we performed in Chapter V, gives an indication of what is happening shortly after infection. But seen the strong regulation of mRNA cytokine expression, it might be also interesting to study effects after longer periods of infection, like 12, 24 and 48 hours. However, this is not possible in this experimental setup due to bacterial overgrowth of the cells and tissues.

Furthermore, our experimental work focused on the reduction of an *E. coli* O157:H7 infection in calves using bLF. We introduced the application of bLF on the

rectal mucosa of cattle to fully benefit from the positive effects of bLF as here, both the direct antibacterial and the immunomodulating effect of bLF can contribute to the reduction of shedding. bLF was first described in the late 1930's (Sorensen & Sorensen 1939) and since then tested and applied in many medical, veterinary and health-promoting applications. Presently, LF is one of the center points in cancer research as it seems to have antineoplastic properties without adverse effects (Yin et al. 2013). But also in animal nutrition, LF used as an additive has shown its importance as it can improve nonspecific immunity and strengthen host defenses in weaned piglets (Wang et al. 2004). bLF is also available on the market in infant and follow-on formulae in European countries, Japan, Indonesia and many others (Morinaga Milk ®). In our study, we were the first to show that bLF can clear an *E. coli* O157:H7 infection in cattle, which is promising. But we recognize that the used rectal application method is inappropriate for treatment on farms, as all animals have to be treated individually rectally on a daily basis. Different strategies to reduce this workload were tested ranging from (1) higher doses of bLF for shorter durations over (2) development of a rectal bLF gel to minimize the number of applications to (3) formulation of pH-sensitive coated bLF pellets which can be mixed in feed. Firstly, a five times higher dose of bLF powder was applied on the rectum of infected animals, but this dose could not accelerate the clearance (Rybarczyk et al. 2015). Secondly, the rectal gel showed comparable results to powder for growth inhibition and bacterial attachment *in vitro*, but had inconsistent effects -possibly due to a weaker mucosal adherence- in a small group of animals (n = 3) *in vivo* (Rybarczyk 2016). Thirdly, the processing technique for coated pellets resulted in a denaturation of 33 % of bLF hampering the biological effects of bLF *in vitro* and was therefore not tested *in vivo* (Rybarczyk 2016). In short, the results of these experiments could not lead to an improvement of the application strategy and new approaches should be elaborated.

A first investigation of the local immune responses in the rectum upon infection and subsequent treatment with bLF powder, disclosed the induction of mucosal IgA responses against EspA and EspB in treated animals only (Rybarczyk et al. 2015). In addition, our cytokine study (Chapter V) also revealed a part of this puzzle: bLF can restore the IL-6 levels which were disarrayed by *E. coli* O157:H7 back to normal in rectal epithelial cells and the RAJ explants. Since IL-6 is important for inducing production of IgA by B-cells (Macpherson et al. 2008), and is known to enhance IgA secretion (Mowat 2003), this might explain how bLF can induce local immune responses.

These findings emphasize the immuno-modulatory effect of bLF in local tissues and performing the RNA-Seq technique on samples from animals infected with *E. coli* O157:H7 and treated with bLF could further assist to elucidate the full picture. A better understanding of the mechanism of clearing and modulation of the immunity might help to identify individual animals or groups of animals at risk. Individual animals which can highly affect the infection status of the herds on a farm are 'supershedders' (Arthur et al. 2013) and identifying and treating these might be a step forward in eliminating *E. coli* O157:H7 from farms. The treatment of other animals in the same herd as a 'supershedder' could also be a possible focus for intervention as we have shown that bLF can result in development of mucosal immune responses which cannot protect from infection but can facilitate the clearance. Three to four months old animals might be part of the focus as well, since they are no longer protected by maternal immunity and rectal immunization with EspA and/or EspB or other virulence factors of *E. coli* O157:H7 in combination with bLF might induce local antibodies as well. It has been shown that EHEC colonization in ruminants is age-dependent and animals between 8 and 18 months old are more often infected, making them also a valuable target for intervention strategies. It might be possible that treating only these animals would significantly reduce the peril of all other animals on the same farm, but for now this is still a distant prospect.

We performed a study to investigate the effect of bLF on Stx-producing strains. In our *in vivo* experiments, we used the Stx-negative *E. coli* O157:H7 NCTC12900 strain for biosafety reasons. Until now, we have no proof that bLF can also reduce or clear shedding of Stx-positive EHEC strains from colonized cattle. Therefore, we analyzed the effect of bLF on purified Stx and on its toxicity in Vero cells in Chapter VI. We observed that bLF could decrease free Stx2, but not Stx1, and that this effect was bLF concentration-dependent. Moreover, we could demonstrate that the receptor-binding B-subunit of Stx2 is degraded by bLF. This is an important finding as strains producing Stx2 are more often associated with severe disease in humans compared to strains that produce only Stx1 or a combination of both toxins (Fuller et al. 2011). We also observed that bLF was able to mitigate the effect of cytotoxic EHEC in our *in vitro* model. This preliminary study is therefore a first step in the investigation of the possible use of bLF in treatment of infected humans, as new strategies are urgently needed. The use of antibiotics is discouraged as antibiotics promote the release of Stx from the bacteria by a cellular stress response, which results in increased cellular toxicity. It is still unclear if

bLF reduces release of the toxin or if it mainly acts by proteolytic degradation, but this might be worth investigating.

In our cytokine study, we could observe a reduction in IL-6 by *E. coli* O157:H7 and supplementation with bLF seemed to restore the IL-6 levels (Chapter V). This effect might be the result of a partial degradation of Stx2 as observed in Chapter VI. However, it remains unclear why IL-8 and TNF-α were not restored to normal levels. Furthermore, we could not observe a clear difference between the $Stx1^{+}Stx2^{+}$ and the $Stx2^{+}$ strain, as would be expected from the different effect of bLF on Stx1 and Stx2.

An important point of consideration is that Stx can also modulate the immune response in cattle. Menge et al. (1999) demonstrated that Stx1 suppresses the mucosa-associated immune response in young calves, while we have suggested that this mucosal immune response plays an important role in the clearance of the bacteria from the gut (Rybarczyk et al. 2015). In our cytokine study (Chapter V) we showed that an exposure to Stx2 followed by infection with an Stx-positive strain can strongly reduce the IL-6 levels, which are important for the local immune response. Therefore, the question remains if bLF will be able to overpower the effect of Stx in the local mucosa. Nevertheless, this multifunctional protein exerts a direct bactericidal effect which is most likely independent of the presence of Stx.

By and large, all these findings suggest that bLF will also affect the colonization of Stx-positive EHEC strains in ruminants, but experiments have to be performed to have an idea of the kinetics of this infection and possible clearance.

In addition, our studies showing the antibacterial effect of bLF and the proteolytic effect on Stx2 open a window of opportunities for new applications. Since we have shown that bLF can clear an infection *in vivo* and that this antibacterial effect does not increase the toxicity of the bacteria in sensitive cells, the use of bLF in human EHEC-cases might be worth considering. Therefore, the effect of bLF on more EHEC-strains should be investigated and more studies on cytotoxicity should be performed.

Summary

Summary

Enterohemorrhagic *Escherichia* coli (EHEC) are zoonotic pathogens that cause several outbreaks associated with human disease every year. Symptoms can range from watery diarrhea to bloody diarrhea and severe hemorrhagic colitis, renal failure and hemolytic uremic syndrome. Cattle, the most important source of transmission to humans, harbor the bacteria in their intestines without showing clinical symptoms. Different studies have tried to map the infection status of farms and of individual animals within herds but this seems to be complex as the infection rates of individual animals can vary widely in time. Cattle do not seem to develop a protective immune response after an initial exposure to the bacteria, making them susceptible for re-infections. It is generally accepted that EHEC is capable of modulating the host immune responses by a variety of factors, which probably causes the absence of a protective immune response. In this thesis, we investigated different aspects of immune modulation by EHEC.

Furthermore, different research groups have tried to reduce the risk of transmission of the pathogen to humans by reducing the carriage rates in cattle. All these strategies showed only limited effects. Therefore, we tested an innovative strategy to reduce EHEC colonization in cattle using bovine lactoferrin (bLF) which combines immunomodulating properties together with antibacterial effects.

Chapter I of this thesis provides a comprehensive overview of the current literature. In order to gain insight in the mechanisms by which EHEC can modulate immune responses, the effects of different immunomodulating EHEC factors are described. Furthermore, the current knowledge on bLF was reviewed in this chapter.

Chapter II describes the aims of the experimental workthat was performed during this PhD thesis. The focus of this thesis to investigate the suppression of the immune response in cattle by *E. coli* O157:H7 and the role of bLF in modifying this suppressed response.

In **Chapter III**, the suppression of the bovine immune system was investigated by RNA-Seq. Calves were experimentally infected and re-infected and RNA was extracted from recto-anal junction (RAJ) tissues, the major colonization site, and ileal Peyer's patches (I+PP) tissues, the gateway to the immune system. This was compared to the

RNA expression in control animals. A striking difference in gene expression was observed in the I+PP after a first infection compared to non-infected control animals. The effects were less pronounced in the RAJ or after a re-infection. These findings motivate the hypothesis of a suppression of the bovine immune response after an initial colonization with *E. coli* O157:H7.

Chapter IV describes the use of bLF as an antibacterial and immunomodulating protein in experimentally infected calves. bLF was applied directly on the rectal mucosa, the most important colonization site, or orally and the effects on systemic immune responses against EHEC type III secretion system (T3SS) proteins EspA, EspB and intimin were investigated. All groups developed serum responses, but no clear differences could be observed between the groups although animals in the rectal group ceased shedding fast and remained negative, whereas no beneficial effect of bLF on bacterial shedding was observed in the oral group. The results indicate that the use of bLF as a rectal treatment can be a useful strategy to preclude further transmission of EHEC infections from cattle to humans. The mechanism behind this effect might be, at least partly, due to stimulation of the local immune responses by bLF, as no systemic effect was observed.

Further research on the modulation of immune responses in cattle was performed in **Chapter V** by analysis of cytokine release (IL-6, IL-8 and TNF-α) in the supernatants of rectal epithelial cells, which were exposed to Shiga toxin 2 (Stx2) and bLF and subsequently inoculated with different *E. coli* O157:H7 strains. The release of pro-inflammatory cytokines and chemokines was also studied in tissue explants from the RAJ and I+PP, incubated with *E. coli* O157:H7, bLF and/or Stx. In rectal epithelial cells, the incubation with *E. coli* O157:H7 strains reduced both IL-6 and IL-8 levels and increased TNF-α levels. bLF was able to restore the IL-6 but not the IL-8 levels and induced the release of supplementary TNF-α. Inoculation of tissue explants with *E. coli* O157:H7 (Stx-) induced the secretion of IL-6 and IL-8 in the RAJ and of IL-8 in the I+PP, while reducing only IL-6 in the I+PP. bLF could again restore IL-6, but overstimulated IL-8 secretion. TNF-α was slightly decreased by *E. coli* O157:H7 (Stx-) in RAJ and increased in I+PP explants. The differences between rectal epithelial cells and RAJ explants support an effect of other (immune) cells, which are only present in the complex explant environment.

Since Chapter IV showed promising results in the reduction of EHEC in cattle and LF is recently in the center of attention as a possible therapy for numerous diseases in humans, **Chapter VI** is a pilot study investigating the possible use of bLF against EHEC infections in humans. Many human cases have described that the use of antibiotics in EHEC infected patients is contra-indicated because of the increased risk for severe outcome due to a higher release of Stx by the bacteria. In this chapter, the non-toxic concentration of bLF for use in Vero-cell cultures was determined. Futhermore, a bLF concentration-dependent decrease of active, cell-free Stx2, but not Stx1 in EHEC cultures was observed. When only colonizing bacteria were taken into account, cytotoxicity could be significantly reduced by 10 and 1 mg/ml bLF during 48 hours. This effect of bLF at least partly results from degradation of the Stx2 receptor-binding B-subunit.

Chapter VII provides a general discussion, highlighting the pro's and contra's of the experimental studies and gives suggestions for future research.The results of the research in this thesis show that *E. coli* O157:H7 can indeed suppress the immune response in cattle. bLF seems to be able to restore disarrayed cytokine expression back to normal levels and was able to clear an *E. coli* O157:H7 infection in calves, probably due to both the antibacterial and immunomodulating effect of the protein. bLF might also become part of a novel treatment strategy of EHEC-infected patients as it does not induce an increased toxicity *in vitro* as seen for antibiotics. However, further research is needed to establish an efficacious treatment.

Samenvatting

Samenvatting

Enterohemorrhagische *Escherichia* coli (EHEC) zijn zoönotische pathogenen die jaarlijks verantwoordelijk zijn voor uitbraken met een aantal ziektegevallen tot gevolg. De symptomen kunnen variëren van waterige diarree tot bloederige diarree en ernstige hemorrhagische colitis, nierfalen en hemolytisch uremisch syndroom. Runderen zijn de belangrijkste bron van overdracht van de bacteriën naar de mens, omdat ze drager zijn van de bacteriën in hun darmen zonder klinische symptomen te vertonen.

Verschillende studies hebben de infectiestatus van bedrijven en van individuele dieren binnen een kudde proberen in kaart brengen, maar dit blijkt heel complex omdat de infectiegraad van individuele dieren sterk kan variëren in de tijd. Runderen lijken geen beschermende immuunresponsen te ontwikkelen na een eerste blootstelling aan de bacteriën, wat hen vatbaar maakt voor herinfecties. Het is algemeen aanvaard dat EHEC in staat is om de immuunresponsen van de gastheer te beïnvloeden door verschillende factoren, dit veroorzaakt waarschijnlijk de afwezigheid van een beschermende immuunrespons. In deze thesis hebben we de verschillende aspecten van immunomodulatie door EHEC onderzocht.

Verder hebben verschillende onderzoeksgroepen geprobeerd om het risico van overdracht van de bacterie naar de mens te verminderen door het verminderen van de hoeveelheid bacteriën in de runderen. Al deze strategieën vertoonden slechts beperkte effecten. Daarom hebben we een innovatieve strategie getest om de EHEC kolonisatie in runderen in te perken. Hiervoor hebben we gebruik gemaakt van boviene lactoferrine (bLF), een eiwit dat immunomodulerende eigenschappen combineert met antibacteriële effecten.

In **hoofdstuk I** van deze thesis werd een uitgebreid overzicht gegeven van de huidige literatuur. Om meer inzicht te krijgen in de mechanismen waardoor EHEC de immuunresponsen kan moduleren, werden de effecten van verschillende immunomodulerende EHEC factoren beschreven. Verder werd ook de huidige kennis m.b.t. bLF in dit hoofdstuk beschreven.

Hoofdstuk II beschrijft de doelen van het experimentele werk dat werd uitgevoerd tijdens dit doctoraatsonderzoek. De focus van deze thesis lag op het onderzoek naar de onderdrukking van de immuunrespons bij runderen door *E. coli* O157:H7 en de rol die bLF kan spelen om deze onderdrukte respons te normaliseren.

In **hoofdstuk III** werd de onderdrukking van het boviene immuun systeem onderzocht door RNA-Seq. Kalveren werden experimenteel geïnfecteerd en geherinfecteerd en RNA werd geëxtraheerd uit weefsels van de recto-anale junctie (RAJ), de belangrijkste kolonisatieplaats, en uit de ileale Peyerse platen (I+PP), de toegangspoort naar het immuunsysteem. Dit RNA werd vergeleken met de RNA expressie van controledieren. Een opvallend verschil in de genexpressie werd gezien in de I+PP na een eerste infectie in vergelijking met niet-geïnfecteerde controle dieren. De effecten waren minder uitgesproken in de RAJ en na een herinfectie. Deze bevindingen ondersteunen de hypothese van een suppressie van de boviene immuunrespons na een initiële kolonisatie met EHEC.

Hoofdstuk IV beschrijft het gebruik van bLF als een antibacterieel en immunomodulerend eiwit in experimenteel geïnfecteerde kalveren. bLF werd rechtstreeks op de rectale mucosa, de belangrijkste kolonisatieplaats, of oraal toegediend en de effecten op de systemische immuunresponsen tegen EHEC type III secretie systeem proteïnen EspA, EspB en intimine werden onderzocht. Alle groepen vertoonden serumresponsen, maar er konden geen duidelijke verschillen tussen de groepen worden gezien. Desalniettemin stopten alle dieren in de rectaal behandelde groep snel met uitscheiden en bleven ze negatief terwijl er geen gunstig effect voor oraal toegediende bLF kon worden gevonden. Deze resultaten tonen aan dat het gebruik van rectaal toegediende bLF een nuttige strategie kan zijn om verdere transmissie van EHEC infecties van runderen naar mensen te verminderen. Het mechanisme verantwoordelijk voor dit effect is waarschijnlijk ten minste deels te wijten aan de stimulatie van lokale immuunresponsen door bLF, aangezien geen stimulatie van systemische responsen werd waargenomen.

Verder onderzoek naar de modulering van immuunresponsen in runderen werd uitgevoerd in **Hoofdstuk V** door de analyse van de vrijstelling van cytokines (IL-6, IL-8 en TNF-α) in het supernatans van rectale epitheliale cellen, die werden blootgesteld aan Shiga toxine 2 (Stx2) en bLF en vervolgens geïnoculeerd met verschillende *E. coli*

O157:H7 stammen. De vrijstelling van proinflammatoire cytokines en chemokines werd ook bestudeerd in weefselexplanten van de RAJ en de I+PP, geïnoculeerd met *E. coli* O157:H7, bLF en/of Stx. In rectale epitheliale cellen verminderde de incubatie met de *E. coli* O157:H7 stammen zowel IL-6 als IL-8 concentraties en verhoogde ze de TNF-α concentraties. bLF kon het IL-6 niveau normaliseren, maar de IL-8 concentratie bleef onveranderd. Voor TNF-α werd een toename waargenomen door bLF. Inoculatie van weefsel explanten met *E. coli* O157:H7 (Stx-) induceerde de vrijstelling van IL-6 en IL-8 in de RAJ en van IL-8 in de I+PP. De IL-6 concentratie nam af in de I+PP. bLF kon opnieuw de IL-6 concentratie normaliseren, maar zorgde voor een overstimulatie van IL-8 secretie. TNF-α werd licht verminderd door *E. coli* O157:H7 (Stx-) in de RAJ en nam toe in de I+PP explanten. De verschillen tussen de rectale epitheliale cellen en de RAJ explanten tonen aan dat er een effect is van andere (mogelijks immuun-) cellen, die alleen aanwezig zijn in het complexe weefsel van de explanten.

Omdat Hoofdstuk IV veelbelovende resultaten aangaf voor de reductie van EHEC in runderen en LF recent in de aandacht is gekomen als mogelijke therapie voor verschillende ziektebeelden bij de mens werd in **Hoofdstuk VI** een pilootstudie uitgevoerd waarbij gekeken werd naar het mogelijke gebruik van bLF tegen EHEC infecties bij de mens. In verschillende patiënten heeft men aangetoond dat het gebruik van antibiotica bij een EHEC-infectie kan leiden tot een verhoogde kans op een verergering van het ziektebeeld door een hogere vrijstelling van Stx door de bacteriën. In dit hoofdstuk werd een niet-toxische concentratie van bLF voor gebruik in Vero-cellijnen uitgetest. Verder werd een bLF concentratie-afhankelijke afname van actieve, cel-vrije Stx2 in EHEC culturen geobserveerd. Wanneer men enkel naar de kolonizerende bacteriën keek, kon de cytotoxiciteit significant verminderd worden door het gebruik van 10 en 1 mg/ml bLF tijdens 48 uur behandeling. Dit effect wordt ten minste deels toegeschreven aan de degradatie van de Stx2 receptor-bindende subeenheid.

Hoofdstuk VII geeft een algemene discussie weer, waarin de voor- en nadelen van de experimentele studies worden besproken en suggesties worden gegeven voor toekomstig onderzoek. De resultaten van het onderzoek in dit doctoraatswerk bevestigen dat *E. coli* O157:H7 de immuun respons in runderen kan onderdrukken. bLF lijkt in staat om de verstoorde cytokine expressie opnieuw te normaliseren en kon ook een *E. coli* O157:H7 infectie in kalveren klaren. Dit is waarschijnlijk te wijten aan zowel het

antibacterieel als het immunomodulerend effect van het eiwit. bLF kan eveneens onderdeel worden van een nieuwe behandelingsstrategie voor EHEC-geïnfecteerde patiënten aangezien dit niet resulteert in een verhoogde toxiciteit zoals voor antibiotica het geval is. Verder onderzoek is nodig om een efficiënte behandeling te ontwikkelen.

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Curriculum Vitae & Bibliography

Curriculum Vitae

Evelien Kieckens was born on October 28th, 1987 in Aalst, Belgium. In 2011, Evelien obtained her Master's degree in Biomedical Sciences, Major Degeneration and Regeneration from the University of Ghent, Belgium. During her master training in the Tissue Engineering Group of the University of Ghent, Evelien worked on dynamic cell culture methods and performed research on cell-biomaterial interactions and cell viability. In Januari 2012, Evelien started her doctoral studies at the Laboratory of Immunology, at the Faculty of Veterinary Medicine of Ghent University, where she spend a 4-years PhD study focusing on the immunomodulation of the bovine host by *E. coli* O157:H7 and the effects of bovine lactoferrin on this modulation. Evelien Kieckens is author and co-author of several publications in peer-reviewed international journals and she has actively participated in several international conferences.

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Participation in international conferences

VTEC 2012, 8th International Symposium on Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections. 6-9 May 2012. Amsterdam, The Nederlands. (participation)

Kieckens E., Rybarczyk J., De Zutter L., Vanrompay D., Cox E. Curative treatment of E. coli O157:H7 infected sheep with lactoferrin. BSFM 20-21 September 2012. Brussels, Belgium. (abstract and poster presentation)

Rybarczyk J., **Kieckens E.**, De Zutter L.,Remon J.P., Vanrompay D., Cox E. Clearance of Escherichia coli O157:H7 infection in calves by rectal administration of bovine lactoferrin. AREA 2013, 5th Symposium on Antimicrobial Resistance in Animals and the Environment. 30 June- 3 July 2013. Ghent, Belgium. (abstract and poster presentation)

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Kieckens E., Rybarczyk J., Barth S., Menge C., Cox E., Vanrompay D. Effects of bovine lactoferrin on release and bioactivity of Shiga toxins from different *E. coli* O157:H7 strains. BSFM 15-16 September 2016. Brussels, Belgium. (poster presentation)

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Italy and also for all the lovely drinks, home-made Italian lunches with and without zia Carmella, dinners, barbecues and free vet-visits for Lola. Now your time has come to change your life again. I wish you and Paolo all the best with your new life and your baby-to-come in Switzerland. I will miss you guys! See you soon when we are coming for a visit and some skiing! **Gosia'ke**, my running mate! Since you are fluently speaking 4 languages, I will try to do the same ☺. J'admire vos compétences d'organisation. Until now it is still unclear to me how you can manage a very busy full time job with 3 children and renovations of your home ☺. Bedankt om er altijd voor ons te zijn met je luisterend oor, zelfs al had je het zelf soms minder makkelijk. Like Ut once said: you are the glue that sticks us together. Jesteś prawdziwym przyjacielem!! (hopelijk heeft Google translate me niet in de steek gelaten met dit laatste ☺) Succes met het laatste deel van je PhD, het einde komt in zicht! Hopelijk kunnen we nog veel (stads)loopkes samen doen. **Michael**, voordat jij kwam was ik het enige Belgje in onze exotische bureau ☺. Ik ben blij dat jij me komt opvolgen en ik wens je alle geluk toe met je PhD! Never give up, je komt er zeker ☺. Veel succes ook met de voorbereidingen van je Belgisch-Nederlandse trouw met Brigitta! **Pedro**, Pedrito, Pedritino, cojones! It has been a while since we saw each other, but I still remember your bull-capturing-throwing-lasso skills like in the movies. I hope our paths will cross again and when they do, we'll go out to eat steak, cojones ☺! **Uttie**, thanks for being our tower of strength in the office. You were there behind your desk full of papers on the first day I arrived and you still are. Although, some of your habits have changed: fish sauce and 'balut' were replaced by broodjes mozzarella, spaghetti and waffles. If you ever go back to Vietnam, let me know so I can send you survival packages with Belgo-Italian food ☺. Thanks for helping me out with the mean blotting-machine ☺! **Yu**, my Chinese friend, I didn't know that Chinese weddings are not about dancing but about playing games. Thanks for making me bridesmaid and good luck back there in China!

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